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STUDIES ON THE PHYSIOLOGY OF *HELMINTHOSPORIUM CARBONUM* ULLSTRUP, THE INCITANT OF CORN LEAF SPOT

by RAM DAYAL *and* M. M. JOSHI,* *Department of Plant Pathology, Banaras Hindu University, Varanasi 5*

(Communicated by R. K. Saksena, F.N.I.)

(*Received 15 January 1968; after revision 24 December 1968*)

The optimum temperature, *p*H and humidity for the growth of *Helminthosporium carbonum* Ullstrup were found to be 26 °C, *p*H 8 and 95 per cent RH respectively. Maltose was the most suitable carbon source for growth, and galactose for sporulation. Tyrosine proved to be the best nitrogen source. Nitrate nitrogen was utilized more efficiently than nitrite and ammonium nitrogen. Of the five hormones tried, none but 3-Indol propionic acid significantly increased the growth and sporulation of the fungus.

INTRODUCTION

Helminthosporium carbonum Ullstrup, the incitant ɟo cornleaf spot, has been found to occur at Varanasi in 1964. From the literature available, it appears that little is known about the physiology of this fungus in culture (Hale and Roane 1961). Therefore, a detailed study on this aspect was undertaken in the present investigation.

MATERIALS AND METHODS

Monosporic culture of *Helminthosporium carbonum* was obtained by single spore isolations from infected corn leaves collected at the College of Agriculture farm, Banaras Hindu University. Stock cultures were maintained on potato —dextrose agar. Asthana and Hawker's medium (Dextrose 5 g, KH_2PO_4 1·75 g, KNO_3 3·5 g, $MgSO_4.7H_2O$ 0·75 g and distilled water 1,000 ml) was used as basal medium throughout the investigation. Twenty-five millilitres of the medium, with the acidity usually adjusted to *p*H 7, were dispensed into 100 ml Pyrex Erlenmeyer flasks and autoclaved at 15 lb pressure for 15 minutes. For all the experiments conducted, inoculum of equal size (5 mm agar discs) was used from 5 to 10 days' old cultures. Unless otherwise indicated, the flasks were incubated at room temperature (24±6 °C). Triplicates were made in each treatment. Amount of growth was based on the oven-dry weight of the washed mycelial mass in each container. The tests on solid media were made in Petri plates (100 mm diameter) containing 20 ml of basal medium with 2 per cent agar. The degree of sporulation was measured by visual observation of the slides examined under microscope. The size of conidia is based on the measurements of 100 conidia and recorded in micron.

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In studies to determine the influence of H-ion concentration of the medium, initial pH values of 3–10 were adjusted with phosphoric acid and sodium hydroxide before autoclaving. To study the effect of humidity, various humidities were maintained by the method suggested by Spencer (1928). Carbon and nitrogen nutrition of the fungus were studied by substituting the original compound, i.e. glucose and potassium nitrate, in the basal medium with different sources of carbon and nitrogen in such a way so as to supply the same amount of these constituents normally present in the basal medium. In the case of hormone trials 10 μg of each hormone was added to 100 ml of the basal medium and 25 ml of this medium was distributed into Erlenmeyer flasks.

RESULTS AND DISCUSSION

Effect of temperature

Environmental factors greatly influence growth, sporulation and conidial size of *Helminthosporium carbonum* Ullstrup. The fungus grew within a temperature range of 18–34 °C, optimum being 26 °C (Table I). This temperature is favourable for sporulation as well as for the size of conidia. Chandwani and Munjal (1963), and Misra and Singh (1963) also observed that best growth of *Helminthosporium gramineum* and *H. turcicum* was obtained at 25° and 25–30 °C respectively.

TABLE I

Radial growth, sporulation and size of conidia of H. carbonum *at different temperatures*

Temperature (°C)	Radial growth* (mm)	Sporulation	Size of conidia (μ)
18	14·0	Good	59·95 × 14·16
22	26·5	Good	62·55 × 15·66
26	57·5	Excellent	69·95 × 15·99
30	30·0	Good	65·99 × 16·23
34	14·5	Fair	41·63 × 11·39

* Growth after 10 days.

Effect of hydrogen-ion concentration

The fungus could grow over a wide range of pH from 3 to 10; a pH range from 6 to 8 was more favourable optimum being pH 8 (Table II). Similar results were obtained by Thind and Sandhu (1956) while working with *Gloeosporium psidii*. The optimum *p*H for growth seems to be best also for sporulation and size of conidia. During the growth period, the fungus considerably increased the initial *p*H of the medium at each level except *p*H 9 and 10, which is in conformity with the results obtained by Hale and Roane (1961) for *Helminthosporium carbonum* Race 1, and Chandwani and Munjal (1963) for *H. gramineum*.

TABLE II

Effect of pH *of the medium on the growth, sporulation and conidial size of* H. carbonum

Initial *p*H of the medium	Final *p*H of the medium	Dry wt. of fungal mat. (mg)	Sporulation	Size of conidia (μ)
3	6·0	19·5	Good	56·61 × 14·15
4	6·1	22·0	Good	63·27 × 16·65
5	6·7	29·3	Good	63·55 × 14·99
6	7·3	32·0	Good	63·71 × 11·65
7	7·6	34·0	Good	65·34 × 21·10
8	8·2	37·3	Good	73·26 × 16·65
9	9·0	34·3	Nil	–
10	10·0	32·6	Nil	–

Effect of relative humidity

The organism under investigation could tolerate a wide range of relative humidity from 55 to 100 per cent. The maximum growth was obtained at 95 per cent RH (Table III). Almost similar results were obtained by Misra

TABLE III

Radial growth, sporulation and size of conidia of H. carbonum *at different relative humidities*

Relative humidity (%)	Radial growth* (mm)	Sporulation	Size of conidia (μ)
100	56·5	Fair	78·2 × 13·99
98	60·75	Good	60·69 × 12·39
95	81·75	Good	55·81 × 12·39
92	74·50	Fair	49·93 × 10·74
88	53·83	Fair	49·90 × 10·72
81	49·50	Fair	44·39 × 10·72
65	49·0	Fair	36·79 × 9·99
55	44·5	Fair	36·63 × 9·99

* Growth after 6 days.

and Singh (1963) for *Helminthosporium turcicum*. There was not much influence of relative humidity on sporulation; however, good sporulation was observed at 95 and 98 per cent RH. Conidia attained maximum size at 100 per cent RH. Similar results were obtained by Klotz (1923), Ramakrishnan (1931), Chaudhury (1944) and Berger (1963) with some of the *Cercospora* spp. studied by them.

Effect of carbon source

Though, disaccharides in general appear to be good sources of carbon for the fungus, maltose was the best followed by galactose (Table IV). Glucose, the common carbon source for many fungi, supported only moderate growth of this fungus. Chlamydospores too were formed on glucose. Fructose seems to be the poorest source. These findings do not agree with those of Hale and Roane (1961) for *H. carbonum* Race 1, but are in conformity with observations reported by Converse (1953), and Chandwani and Munjal (1963) for *Helminthosporium gramineum*. The difference in the utilization of sugars by *H. carbonum* may be attributed to the behaviour of different isolates of the fungus. Starch was also utilized as a sole source of carbon and this suggests the possibility of production of amylase enzyme capable of hydrolysing it. Similar result was found with *H. gramineum* by Chandwani and Munjal (1963). No growth was observed in complete absence of carbon. The carbon source which is best for growth may not always prove good for sporulation. Excellent sporulation and largest conidia were observed on galactose while no sporulation occurred on maltose. Sucrose supported poor sporulation. Sucrose and maltose supported poor or no sporulation of *Leptomitus lacteus* (Schade 1940) and *Chytridium* sp. (Crasemann 1954) as well.

TABLE IV

Effect of different sources of carbon on growth, sporulation and size of conidia of H. carbonum

Carbon sources		Dry wt. of fungal mat. (mg)	Sporulation	Size of conidia (μ)
Glucose	..	53·6	Good	41·62 × 10·72
Fructose	..	40·6	Poor	49·95 × 10·72
Galactose	..	78·3	Excellent	78·25 × 14·98
Sucrose	..	73·0	Fair	49·95 × 10·72
Lactose	..	73·0	Good	46·62 × 9·57
Maltose	..	85·0	Nil	–
Starch	..	47·0	Poor	36·63 × 9·99
Control	..	0·0	Nil	–

Effect of nitrogen source

Since the fungus under study showed very little growth and no sporulation in the absence of nitrogen, it is evident that nitrogen is essential for its growth and sporulation (Table V). Good utilization of nitrate nitrogen (given in the form of potassium and sodium nitrate) finds support from the results obtained by Thind and Sandhu (1956), and Misra and Thakur (1965) for *Gloeosporium psidii* and *G. musarum* respectively. Sodium nitrite was comparatively a poor source and in this respect the results were similar with that of Saksena and Kumar (1960) for some *Sphaeropsidales*. Ammonium sulphate proved to be the poorest source of nitrogen. This may be due to a sharp drift in *p*H towards acidic side. Similar results were obtained by Blank and Talley (1941) with *Phymatotrichum omnivorum*, Thind and Randhawa (1957) with *Colletotrichum capsici*, and Singh and Khanna (1966) with citrus isolate of *Alternaria tenuis*. Among the organic sources used, tyrosine supported best growth. Asparagine also proved to be a good source of nitrogen while methionine was poorest among all the organic sources used. The above findings agree with those of Converse (1953), and Chandwani and Munjal (1963) for *Helminthosporium gramineum*. All the nitrogen sources, except methionine, supported good sporulation. Only abundant chlamydospores were formed on ammonium sulphate and in the medium devoid of nitrogen, which were most unfavourable for growth. Maximum size of conidia was obtained on sodium nitrate.

TABLE V

Effect of different sources of nitrogen on growth, sporulation and conidial size of H. carbonum

Nitrogen source	Dry wt. of fungal mat. (mg)	Sporulation	Size of conidia (μ)
Sodium nitrite	29·0	Good	44·95 × 10·82
Sodium nitrate	69·0	Good	61·60 × 11·65
Potassium nitrate	60·0	Good	54·94 × 14·15
Ammonium sulphate	23·3	Nil	–
Asparagine	80·0	Good	59·94 × 14·98
Glycine	61·0	Good	48·28 × 15·81
Leucine	62·0	Good	59·94 × 14·98
Methionine	24·0	Fair	46·62 × 11·65
Tyrosine	89·5	Good	58·27 × 14·98
Control	12·0	Nil	–

Effect of hormones

Five growth regulators, namely 3-Indol propionic acid (IPA), Indol-3-yl-acetic acid (IAA), 1-Naphthyl acetic acid (NAA), Indolyl-3-butyricum acetic acid (IBA) and 2, 4-Di chlorophenoxy acetic acid (2, 4-D), were tried for their effects on growth and sporulation.

The results (Table VI) indicate that except 3-Indol propionic acid none of the other hormones could influence the growth of the fungus; however, Indolyl-3-butyricum acetic acid considerably reduced mycelial output. Almost similar results were found by Thind and Sandhu (1956) for *Gloeosporium psidii*. The reduction by some hormones in the dry weight of this fungus may be attributed to concentration used in this study.

TABLE VI

Growth, sporulation and size of conidia of H. carbonum *in the presence of different growth regulators in basal medium*

Growth regulator	Dry wt. of fungal mat. (mg)	Sporulation	Size of conidia (μ)
IPA	40·0	Excellent	58·27 × 14·98
IAA	26·0	Nil	–
NAA	30·0	Good	58·27 × 13·32
IBA	16·3	Nil	–
2, 4-D	28·0	Nil	–
Control	28·6	Good	59·94 × 14·15

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* Original not seen.

UTILIZATION AND SYNTHESIS OF OLIGOSACCHARIDES BY SOME PATHOGENIC ISOLATES OF *COLLETOTRICHUM GLOEOSPORIOIDES* PENZ.

by Bihari Lal *and* R. N. Tandon, F.N.I., *Department of Botany, University of Allahabad, Allahabad*

(*Received 16 March 1968; after revision 5 December 1968*)

The paper includes the chromatographic studies on utilization of four oligosaccharides (viz. maltose, sucrose, lactose and raffinose) as well as mixtures of their hydrolytic products by five isolates of *Colletotrichum gloeosporioides* Penz. obtained from leaf-spots of *Artocarpus heterophylla* Lam. (Isolate A), *Annona squamosa* L. (Isolate B), *Mussaenda frondosa* Linn. (Isolate C), *Codiaeum variegatum* Blume (Isolate D) and *Manihot esculenta* Crantz. (Isolate E). Sucrose, maltose and raffinose were used up through hydrolytic pathway. Only in one case (Isolate E) hydrolytic product of lactose could be traced. The organisms were also capable of using the hydrolytic products of oligosaccharides. All the isolates synthesized one/two oligosaccharide(s) during the utilization of sucrose and maltose respectively. They failed to do so during the assimilation of lactose and raffinose. Growth of all the pathogens was better on sucrose than on a mixture of glucose and fructose. They exhibited inferior growth on maltose than on glucose. Isolates A, B and E showed superior growth on glucose-galactose mixture than on lactose; isolate C produced better growth on lactose than on its hydrolytic products, while isolate D revealed on special preference. When grown on mixture of glucose-fructose-galactose and raffinose, two isolates (B and D) showed better growth, and the remaining isolates exhibited inferior growth on the mixture than on raffinose.

Introduction

The oligosaccharides are complex sugars composed of two or more monosaccharides linked together by glycosidic bonds. These sugars are water soluble and on hydrolysis they yield monosaccharides. When a hydroxyl group of one monosaccharide unites with the reducing group of another a disaccharide is formed. In a similar manner tri-, tetra- and higher saccharides are formed by condensation. The glycosidic linkage of the oligosaccharide must be broken in order that the oligosaccharide may be available for metabolic transformation. Fruton and Simmonds (1958) have illustrated that in a biological system this cleavage is brought about with the help of enzymes by two general mechanism. The first involves the hydrolysis and the second the phosphorolysis of a glycosidic bond.

A large number of fungi including *Aspergillus niger* (Bacon and Bell 1953), *Penicillium spinulosum* (Bealing and Bacon 1953), *Aspergillus* spp.

(Aso and Shibasaki 1953), *Penicillium chrysogenum* (Giri *et al.* 1953), *Aspergillus flavus* (Giri *et al.* 1954), *Saccharomyces fragilis* (Robert and McFarren 1953), *Myrothecium verrucaria* (Cook and Stone 1953), *Phyllosticta* spp. (Bilgrami and Tandon 1957), *Pestalotia banksiana* and *P. citri* (Tandon and Bilgrami 1958), *Aspergillus* spp. (Mehrotra and Agnihotri 1961), *Cercosporina ricinella*, *Colletotrichum gloeosporiodes* and *Curvularia penniseti* (Tandon and Chandra 1962), *Alternaria tenuis*, *Colletotrichum capsici* and *C. gloeosporioides* (Chaturvedi 1961), *Macrophomina phaseoli*, *Fusarium solani* and *Botryodiplodia ananassae* (Bhargava 1962), *Colletotrichum gloeosporioides*, *Pestalotia pauciseta* and *Botryodiplodia theobromae* (Prasad 1963), *Colletotrichum gloeosporioides* and *C. dematium* (Ghosh 1964), *Trichothecium roseum*, *Curvularia lycopersici* and *Fusarium semitectum* (Kakkar 1964), *Colletotrichum gloeosporioides*, *C. papayae*, *Gloeosporium psidii* and *G. musarum* (Ghosh *et al.* 1965) and *Botryodiplodia theobromae* (Srivastava 1966) are known to synthesize new oligosaccharides when cultured on media with di- or trisaccharides. Recent researches have suggested that the so-called hydrolytic enzymes which cause the hydrolysis of the oligosaccharides can also cause the replacement reaction of the glycosidic bond resulting in the formation of a new oligosaccharide.

In nature, the oligosaccharides exist either as such or as the unit of polysaccharides. Occasionally these oligosaccharides are formed on partial hydrolysis of polysaccharides. They are, therefore, quite important in the nutrition of fungi. The present investigation was undertaken in order to determine (1) the ability of five isolates of *C. gloeosporioides* to utilize the oligosaccharides, (2) their pathway of utilization (indirect or direct) as well as (3) the probable effect of hydrolytic products on growth.

MATERIALS AND METHODS

Single spore cultures of *Colletotrichum gloeosporioides* Penz. isolated from leaf-spots of *Artocarpus heterophylla* (Isolate A), *Annona squamosa* (Isolate B), *Mussaenda frondosa* (Isolate C), *Codiaeum variegatum* (Isolate D) and *Manihot esculenta* (Isolate E) were employed. They were grown on a number of media and on the basis of the results it was decided to use glucose, 10·0 g; KNO_3, 3·5 g; KH_2PO_4, 1·75 g; $MgSO_4 . 7H_2O$, 0·75 g and distilled water, 1000 ml as the basal medium. In order to study the effect of various oligosaccharides (viz. maltose, sucrose, lactose and raffinose) and the mixture of their hydrolytic products they were suitably substituted for glucose and their quantity was so adjusted as to furnish 4 g of carbon per litre. The most suitable *p*H for isolates B, C and E was found to be 6·0 while for A and D it was 5·0 and 5·5 respectively. The *p*H of media for subsequent studies was adjusted to the most suitable level in each case. Other methods used were similar to those mentioned by Lal and Tandon (1968).

OBSERVATIONS AND DISCUSSION

The data have been summarized in Tables I–IV. Regarding the utilization of oligosaccharides by fungi two theories have been suggested. According to Emil Fischer, the oligosaccharides are first hydrolysed with the help of enzymes into their component sugars and are then assimilated. Thus the failure to utilize an oligosaccharide may be due to the inability of the organism to synthesize the necessary hydrolytic enzymes. According to Richard Willstatter, oligosaccharides are utilized by a pathway that does not require hydrolysis before utilization (*see* Lilly and Bernett 1953). The results of the present study support the first view as all the sugars were utilized through indirect pathway.

Assimilation of sucrose (Table I)

Sucrose was readily utilized by all the fungi through the hydrolytic pathway and in each case the hydrolytic product glucose was used up earlier than fructose. A similar result was obtained when the pathogens were allowed to grow on mixture of glucose and fructose. All of them synthesized two oligosaccharides in the media containing sucrose. A simultaneous synthesis of oligosaccharides, along with the hydrolytic products in the medium containing this sugar, has also been reported by several authors. *Colletotrichum inamdarii* (Hasija 1965), however, failed to produce any synthetic oligosaccharide on sucrose.

The dry weights of all the pathogens on sucrose were maximum on 11th day, after which they decreased. They showed continuous increase in dry weight up to the end of the incubation period on mixture of glucose-fructose. Final dry weights of all the isolates were inferior on glucose-fructose mixture than on sucrose. Bhargava (1962) observed that dry weights of *Macrophomina phaseoli* and *Botryodiplodia ananassae* on glucose-fructose mixture and sucrose were similar, though the final dry weight of *Fusarium solani* was inferior on glucose-fructose mixture than on sucrose alone. According to Lilly and Barnett (1951), 'A complex carbohydrate and its hydrolytic products are not necessarily equivalent in all respects.'

Assimilation of lactose (Table II)

The rate of utilization of lactose was very slow, as all the isolates were unable to finish it even at the end of the incubation period of 15 days. None of them synthesized any oligosaccharide. Similar results were obtained for *Colletotrichum gloeosporioides* (Tandon and Chandra, Ghosh *et al.*), *C. capsici* (Chaturvedi) and *C. papayae* (Ghosh *et al.*). On the other hand, *C. gloeosporioides* (Prasad) and *C. inamdarii* (Hasija) utilized lactose in 14 and 12 days respectively. Only isolate E could partially hydrolyse lactose after 8 days and galactose was observed in the medium for 3 days. Srivastava

TABLE I

Showing average dry weight (mg), final pH *and presence of different sugars during the utilization of sucrose and a mixture of glucose and fructose by different isolates of* C. gloeosporioides

Organisms	Sugar	5 days		10 days		15 days		Presence of sugars (days)				
		Dry weight	Final *p*H	Dry weight	Final *p*H	Dry weight	Final *p*H	sucrose (Rf. 0·41)	glucose (Rf. 0·59)	fructose (Rf. 0·63)	oligo-saccharide I (Rf. 0·35)	oligo-saccharide II (Rf. 0·26)
Isolate A ..	sucrose	54·0	6·7	126·4	7·0	103·0	7·6	0–3	1–4	1–7	2–6	3–4
	½ glucose + ½ fructose	50·5	6·1	70·0	6·4	88·3	7·0	–	0–5	0–15	–	–
Isolate B ..	sucrose	49·9	6·7	143·0	7·0	101·9	7·3	0–5	3–6	3–8	2–10	3–7
	½ glucose + ½ fructose	15·5	5·5	50·0	6·1	61·0	7·3	–	0–10	0–15	–	–
Isolate C ..	sucrose	40·4	5·8	111·2	7·0	88·4	7·6	0–5	3–6	3–8	2–7	3–6
	½ glucose + ½ fructose	27·0	6·1	67·5	6·4	82·0	7·3	–	0–9	0–15	–	–
Isolate D ..	sucrose	30·0	6·7	115·0	6·7	89·6	7·3	0–4	1–6	1–8	1–6	2–6
	½ glucose + ½ fructose	22·5	5·5	55·0	6·1	60·6	6·4	–	0–8	0–14	–	–
Isolate E ..	sucrose	41·4	7·0	102·7	7·0	87·6	8·0	0–3	2–5	2–7	2–5	3–5
	½ glucose + ½ fructose	33·0	6·1	81·3	7·3	85·5	6·7	–	0–5	0–15	–	–

TABLE II

Showing average dry weight (mg), final pH *and presence of different sugars during the utilization of lactose and mixture of glucose and galactose by different isolates of* C. gloeosporioides

Organisms	Sugar	5 days		10 days		15 days		Presence of sugars (days)		
		Dry weight	Final *p*H	Dry weight	Final *p*H	Dry weight	Final *p*H	lactose (Rf. 0·43)	glucose (Rf. 0·61)	galactose (Rf. 0·57)
Isolate A	lactose	6·6	5·8	15·0	5·8	17·2	6·1	0–15	–	–
	½ glucose + ½ galactose	48·0	6·7	92·0	7·0	98·5	7·0	–	0–3	0–13
Isolate B	lactose	6·0	5·8	11·8	6·1	22·8	6·7	0–15	–	–
	½ glucose + ½ galactose	18·0	5·8	45·6	6·4	66·0	7·0	–	0–7	0–10
Isolate C	lactose	15·3	6·1	75·9	6·4	75·2	7·0	0–15	–	–
	½ glucose + ½ galactose	28·3	6·1	44·6	6·4	57·6	7·0	–	0–7	0–15
Isolate D	lactose	12·3	5·8	20·0	6·1	50·8	6·4	0–15	–	–
	½ glucose + ½ galactose	22·6	5·5	45·6	6·4	51·3	6·7	–	0–4	0–14
Isolate E	lactose	8·7	5·8	18·5	6·1	26·1	6·7	0–15	–	8–10
	½ glucose + ½ galactose	36·3	6·1	50·3	6·7	73·3	7·3	–	0–9	0–14

(1965), while working with *Curvularia verruculosa* and *C. trifolii*, also detected galactose as the hydrolytic product of lactose in the medium. The other hydrolytic product (viz. glucose) was not detected in the medium. It may be due to slow breakdown of lactose and simultaneous utilization of glucose by the pathogen. This indicated that β-galactosidase responsible for splitting of lactose was produced by the organism. The remaining four isolates, however, failed to produce the hydrolytic products in sufficient quantity. It is possible that they might have been used up simultaneously. Kakkar observed that even when there was no trace of hydrolytic products (viz. glucose and galactose) *Curvularia lycopersici* synthesized an oligosaccharide on lactose medium. Srivastava (1965) also reported oligosaccharide synthesis on lactose by *Curvularia verruculosa*, *C. pallescence* and *C. fallax*.

When grown on a mixture of glucose-galactose, all the organisms under study consumed glucose earlier than galactose, and their dry weight yields showed an increase up to the end of the incubation period. Except for *C. gloeosporioides* isolates C and D, the dry weights were superior on a mixture of glucose-galactose than on lactose. Mehrotra and Kumar (1962) obtained similar results with *Penicillium* spp. The difference was negligible in isolate D, being similar to three isolates of *C. gloeosporioides* and two isolates of *C. dematium* studied by Ghosh. Isolate C differed from the others in that the dry weight was greater on lactose than on the mixture of glucose and galactose.

Assimilation of maltose (Table III)

Maltose was used up by all the isolates through hydrolytic pathway. Although glucose, the hydrolytic product, could be detected only in the medium used by isolates C and E, an oligosaccharide was synthesized in every case which was an evidence of the breakdown of this sugar. Non-appearance of glucose in those cases may be due to its simultaneous utilization or to its conversion to an oligosaccharide. The breakdown of maltose indicated the presence of α-glycosidase in all the isolates of *Colletotrichum*.

Chaturvedi, Tandon and Chandra as well as Prasad have also reported synthesis of an oligosaccharide by their isolates of *C. gloeosporioides* in maltose medium. Other isolates of the same species studied by Ghosh and Ghosh *et al.*, however, synthesized two oligosaccharides. *Colletotrichum capsici* (Chaturvedi) and *C. inamdarii* (Hasija) were altogether different in this respect as they did not show the formation of any oligosaccharide in maltose medium.

After 10 days of incubation, only isolate B showed a slight increase in dry weight, whereas the remaining four isolates exhibited a decrease. All the isolates grew better on glucose (hydrolytic product) than on maltose. Tandon and Chandra obtained similar results with *Colletotrichum gloeosporioides* and *Curvularia penneseti*, but they observed approximately similar growth of

TABLE III

Showing average dry weight (mg), final pH *and presence of different sugars during the utilization of maltose and glucose by different isolates of* C. gloeosporioides

Organisms	Sugar	5 days		10 days		15 days		Presence of sugars (days)		
		Dry weight	Final *p*H	Dry weight	Final *p*H	Dry weight	Final *p*H	maltose (Rf. 0·40)	glucose (Rf. 0·59)	oligo-saccharide I (Rf. 0·28)
Isolate A	maltose	30·4	6·1	91·6	6·7	74·0	7·3	0–10	–	5–10
	glucose	40·4	6·1	110·4	6·4	98·8	7·3	–	0–8	–
Isolate B	maltose	23·0	6·1	52·9	6·7	62·0	7·3	0–10	–	4–10
	glucose	52·8	6·7	122·5	7·0	110·0	7·3	–	0–8	–
Isolate C	maltose	54·1	6·1	99·6	6·7	78·2	7·3	0–6	3–7	3–6
	glucose	67·6	7·0	122·4	7·0	116·0	7·3	–	0–7	–
Isolate D	maltose	40·5	6·1	71·0	6·7	57·4	7·3	0–10	–	4–10
	glucose	42·1	6·1	109·1	6·7	99·6	7·3	–	0–8	–
Isolate E	maltose	37·0	6·1	71·8	6·7	56·9	7·3	0–9	4–10	3–10
	glucose	58·4	6·7	116·4	7·0	106·4	7·3	–	0–8	–

TABLE IV

TABLE IV

Showing average dry weight (mg), final pH *and presence of different sugars during the utilization of raffinose and a mixture of glucose, fructose and galactose by different isolates of* C. gloeosporioides

Organisms	Sugar	5 days		10 days		15 days		Presence of sugars (days)				
		Dry weight	Final *p*H	Dry weight	Final *p*H	Dry weight	Final *p*H	raffinose (Rf. 0·31)	melibiose (Rf. 0·35)	fructose (Rf. 0·65)	galactose (Rf. 0·57)	glucose (Rf. 0·61)
Isolate A ..	raffinose	28·8	5·8	45·5	6·1	76·6	7·3	0–9	–	3–11	3–13	–
	$\frac{1}{3}$ glucose + $\frac{1}{3}$ fructose + $\frac{1}{3}$ galactose	24·0	6·1	84·6	7·0	74·5	7·0	–	–	0–10	0–10	0–4
Isolate B ..	raffinose	8·0	5·8	15·8	6·7	22·5	7·3	0–15	9–15	–	–	–
	$\frac{1}{3}$ glucose + $\frac{1}{3}$ fructose + $\frac{1}{3}$ galactose	20·0	5·8	50·6	6·1	60·5	6·4	–	–	0–15	0–15	0–7
Isolate C ..	raffinose	23·0	5·8	35·5	6·1	75·0	7·0	0–12	–	–	4–13	–
	$\frac{1}{3}$ glucose + $\frac{1}{3}$ fructose + $\frac{1}{3}$ galactose	18·3	6·1	60·5	6·4	66·5	7·0	–	–	0–15	0–15	0–7
Isolate D ..	raffinose	16·3	5·8	29·0	6·1	59·0	6·7	0–15	5–14	–	4–15	–
	$\frac{1}{3}$ glucose + $\frac{1}{3}$ fructose + $\frac{1}{3}$ galactose	15·0	5·8	59·5	6·7	67·3	7·0	–	–	0–7	0–15	0–5
Isolate E ..	raffinose	29·0	6·1	47·8	6·1	52·8	6·7	0–14	–	5–13	5–14	–
	$\frac{1}{3}$ glucose + $\frac{1}{3}$ fructose + $\frac{1}{3}$ galactose	10·3	6·1	32·0	6·4	44·0	7·0	–	–	0–15	0–15	0–8

Cercosporina ricinella on media containing glucose/maltose. Mehrotra and Agnihotri working with five species of *Aspergillus* noticed that the growth was better on maltose than on glucose.

Assimilation of raffinose (Table IV)

The different isolates of the same species showed marked variation in the utilization of raffinose. Two isolates (B and D) could not consume it even in 15 days, while isolates A, C and E finished it in 9, 12 and 14 days respectively. Only one hydrolytic product, galactose, was detected in the medium used by isolate C, while fructose and galactose were detected in medium used by isolates A and E. Melibiose was detected in isolate B only. Isolate D developed both melibiose and galactose and in this respect it was like *C. gloeosporioides* studied by Ghosh *et al.* The presence of melibiose in the medium indicated that splitting of raffinose by isolates B and D took place at the β-linkage and enzyme β-glycosidase was produced. In other cases actual pathway of breakdown of raffinose could not be established.

During the utilization of raffinose by the isolates of *Colletotrichum* all the specific products of hydrolysis were not always detected, although the presence of some product(s) in the medium indicated that hydrolysis had taken place. The failure of the hydrolytic products to appear in the medium has been attributed to their simultaneous utilization. Bhargava and Prasad, working with *Fusarium solani* and *Colletotrichum gloeosporioides* respectively, reported that raffinose was utilized directly by these fungi since none of its hydrolytic products could be detected in the medium.

None of the isolates could synthesize oligosaccharide during the utilization of raffinose. In this respect their behaviour was similar to the isolate of the same species studied by Chandra, Prasad and Ghosh. *Colletotrichum papayae* (Ghosh *et al.*) was different as it synthesized an oligosaccharide in the medium during the utilization of raffinose.

When the organisms were grown on a mixture of glucose, galactose and fructose, it was observed that in every case glucose was used up earlier than galactose and fructose. Fructose was consumed earlier than galactose by isolate D, while isolate A utilized fructose and galactose simultaneously in 10 days. The remaining isolates failed to consume fructose and galactose up to the end of the incubation period of 15 days.

The dry weights of all the pathogens under study continued to increase up to the end of the incubation period. When the pathogens were grown on a mixture of glucose, galactose and fructose, two isolates (B and D) showed better growth on it than on raffinose. In this respect they were like *Nephalium* isolate of *Colletotrichum gloeosporioides* (Prasad). Three remaining isolates exhibited inferior dry weights on a mixture of glucose, galactose and fructose than on raffinose.

CONCLUSION

It is evident from the above results that different isolates of the same species showed marked variation in the utilization of various oligosaccharides as well as their component sugars. Sterilization of the media containing these oligosaccharides did not show any breakdown product but the appearance of monosaccharides in such cases during the growth of various isolates appears to be due to enzymatic activity of these fungi. It was also confirmed that organisms which could assimilate a given oligosaccharide were also capable of using its hydrolytic products.

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STUDIES IN THE PROTEACEAE

XIII. TRIBE GREVILLEEAE

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The author revised Brown's Grevilleeae (20 genera) and limited the tribe to 7 genera with zygomorphic flowers and biovulate carpels. The morphology of the vegetative and reproductive parts, floral anatomy and embryology of some species of all the 7 genera are described. The evolutionary trends within the tribe are discussed and they are correlated with the known chromosome numbers. Since the most primitive genera of the tribe (*Euplassa*, *Gevuina*) resemble the Persoonieae, the latter are considered to be ancestral to Grevilleeae. Several of the evolutionary trends of the family attain their culmination within this tribe. All evidences point to the origin of the tribe under rain-forest conditions and their migration into open and arid regions. On the basis of species concentration and geographic distribution, East Australian region on a southern continent (Gondwanaland) is fixed as the probable centre of origin for the tribe and their present-day disjunct distribution is attributed to continental drift.

INTRODUCTION

The sub-family Grevilleoideae was divided by Brown (1810) into 3 tribes, the Banksieae (2 genera), Embothrieae (11 genera) and the Grevilleeae (20 genera). This classification was accepted by Bentham (1870) and Engler (1894). However, while Banksieae and Embothrieae appear somewhat well defined due to the presence of ellipsoidal pollen grains and multiovulate carpels respectively, the Grevilleeae seem to be a heterogeneous assemblage, comprising taxa with regular as well as zygomorphic flowers and biovulate as well as multiovulate carpels. In the revised classification, the author transferred the two genera with 4-ovuled carpels (*Darlingia* and *Buckinghamia*) to Embothrieae (*sensu lato*), raised *Orites* and *Lambertia* to the rank of separate tribes (Venkata Rao 1957, 1968*b*) and split the remaining taxa into two tribes, the Macadamieae (9 genera with regular flowers) and Grevilleeae (7 genera with zygomorphic flowers).

Brough (1933) and Kausik (1942) studied the life history of *Grevillea robusta* and the latter author described vermiform endosperm haustorium; Kausik (1941) and Haber (1959) studied the floral anatomy of a few taxa. The author described the morphology, floral anatomy and embryology of several species of *Grevillea* and *Hakea* and also the polymorphic pollen grains of *G. vestita* (Venkata Rao 1964, 1966*a*, 1966*b*, 1968*a*). The chromosome numbers in a few taxa have been reported by the author (Venkata Rao 1957), Ramsay (1963) and Johnson and Briggs (1963). Details regarding the numbers of species in each genus, their geographic distribution and the known chromosome numbers are given in Table I.

TABLE I

No.	Genus	*n*	2*n*	Author(s)	Number of species	Geographic distribution
1	*Euplassa* Salisb.				20	S. America
2	*Gevuina* Molina.		28	J and B*	3	1 E. Australia 1 New Guinea 1 Chile
3	*Kermadecia* Brongn. and Gris.				10	7 New Caledonia 2 Fiji, Samoa 1 New Hebrides
4	*Finschia* Diels.				4	New Guinea New Hebrides Palau, Aru
5	*Strangea* Meissn.		22	J and B	3	1 E. Australia 2 W. Australia
6	*Grevillea* R. Br.	10	20	VR,* R*	263	14 New Caledonia 2 New Guinea 1 Tasmania, rest East and West Australia
7	*Hakea* Schrad.	10	20	VR, R	124	c. 80 W. Australia 40 E. Australia 7 Tasmania of which 4 are common with E. Australia

* J and B = Johnson and Briggs (1963); VR = Venkata Rao (1957); R = Ramsay (1963).

MATERIALS AND METHODS

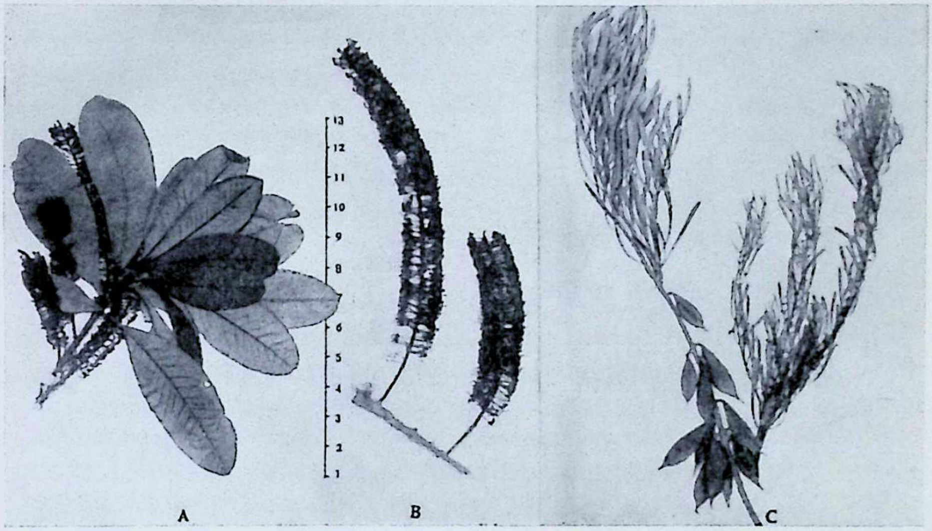
This paper describes the morphology, floral anatomy and embryology of some species of all the 7 genera of the Grevilleeae. Both herbarium specimens and materials fixed in formalin-acetic-alcohol were studied. Customary methods of microtechnique were followed and the materials were embedded in paraffin wax of 60 °C m.p. Sections were cut from 6 μ to 10 μ in thickness and stained in Delafield's haematoxylin or a combination of crystal violet and erythrocin.

VENKATA RAO. *Proc. natn. Inst. Sci. India*, Vol. 35, B, Plate XXXVIII

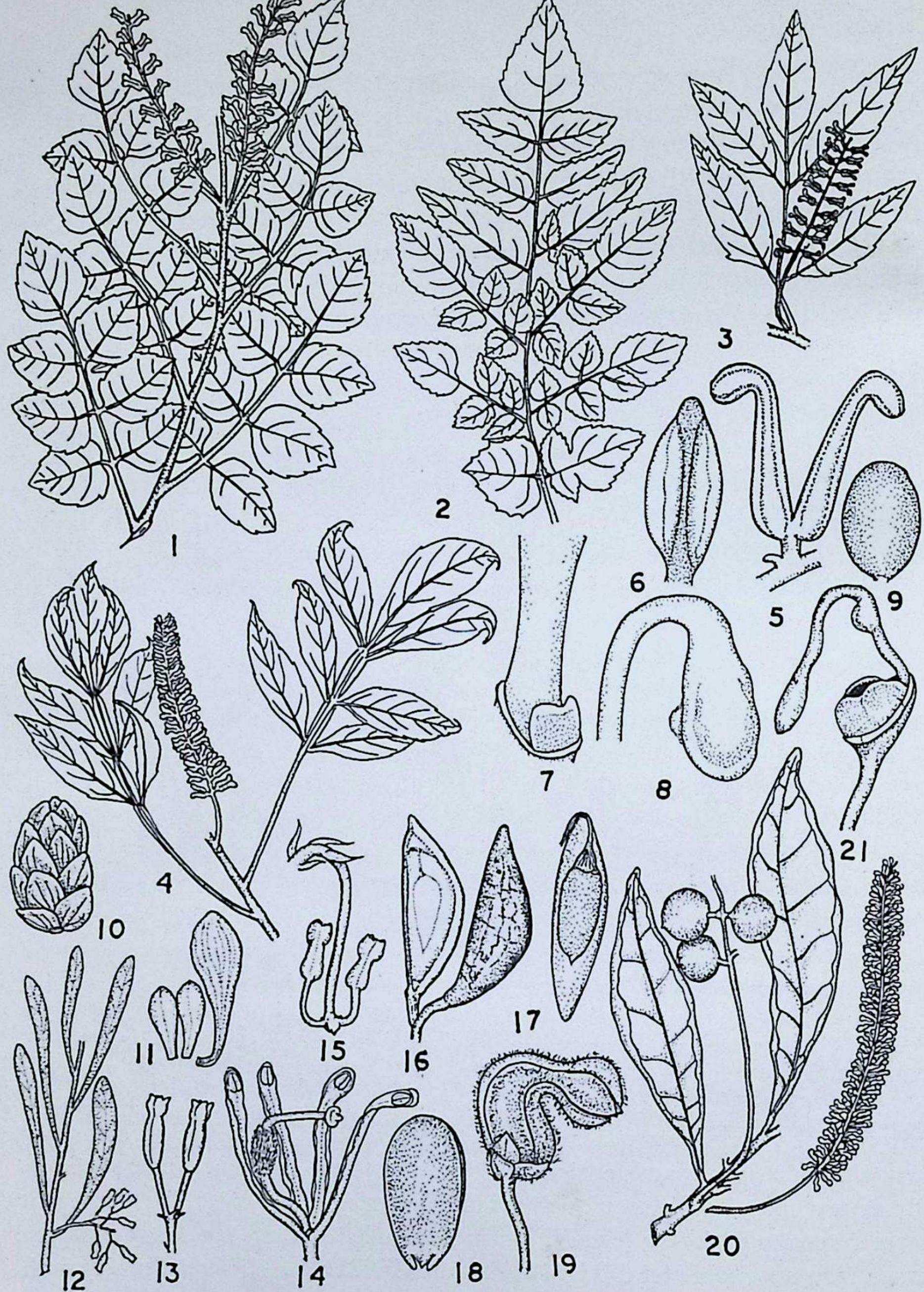
OBSERVATIONS

External morphology

The Grevilleeae show much diversity in habit and morphological features of vegetative and reproductive parts. Sleumer (1955) describes that species of *Finschia* inhabiting the rain-forests and marshy regions of New Guinea are stately trees with buttressed stems and stilt roots. *Kermadecia vitiensis, Grevillea robusta, Hakea laurina, Gevuina avellana* and *Euplassa duquei* are also trees. Shrubs are very common; a few taxa like *Grevillea australis* and *Hakea corymbosa* are undershrubs. The largest leaves (in the tribe as well as in the family) are seen in *Gevuina avellana* and *Grevillea robusta* in which they are bicompound and measure about 35 × 25 cm. (Fig. 2). Pinnately compound leaves are found in species of *Gevuina, Euplassa, Kermadecia vitiensis, Grevillea banksii* and *Hakea adnata* (Figs. 1, 3, 4). In *Gevuina papuana* the leaves are generally compound with winged petioles, but show transition to lobed and entire ones on the same branch. Large simple leaves are seen in *Hakea victoriae* and *Kermadecia sinuata*. The leaves in several species of *Hakea* (e.g. *H. laurina*) and all the three species of *Strangea* are narrow and isobilateral (Fig. 12; Plate XXXVIII C). In *Grevillea shepherdi* and some species of *Hakea* (*H. acicularis, H. microcarpa*) they are terete and markedly xerophytic. The culmination of reduction in the leaf is noticed in *Grevillea intricata* and *G. juniperina* in which they are microphyllous and



A—a branch of *Finschia carryi* with inflorescences.
B—a twig of *Finschia chloraxantha* with cauliflorous inflorescences.
C—a branch of *Strangea linearis* with inflorescences and old fruits.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FIGS. 1–21.

spinescent. *Hakea trifurcata* is interesting in showing dimorphism of leaf: expanded and terete leaves occur on the same branch.

The inflorescences in *Grevillea robusta* and *Kermadecia vitiensis* are large terminal panicles; in *K. sinuata* and *Finschia chloraxantha* they are cauliflorous (Plate XXXVIII B). More commonly they are uniaxial pseudoracemes (Figs. 1, 3, 4, 12, 20; Plate XXXVIII A, B). In *Grevillea robusta* the flowers sometimes occur in groups of three or four at a node showing that the paired flower arrangement which is characteristic of all Grevilleoideae has not become fixed in this species. The inflorescence in *Hakea laurina* is a globose umbel of 200–300 flowers. In several species of *Hakea* and some species of *Grevillea* (*G. australis*, *G. linearis*) it is a small, few-flowered corymb. The three species of *Strangea* show interesting stages of reduction in the inflorescence; in *S. linearis* it is a lax panicle in which there are peduncles for flower pairs, pedicels for flowers, common as well as individual bracts for the flowers; some extra bracts are also found which show that reduction of some flower pairs has occurred (Figs. 12, 13). In *S. stenocarpoides* the peduncle shows two or three empty bracts at base and 2–3 flowers at the top placed singly at each node; there are neither common nor individual floral bracts (Fig. 15). In *S. cynanchicarpa* the short peduncle terminates in a single sessile flower which is surrounded at base by an involucre of 5–6 bracts (Fig. 19). Similar 1-flowered involucrate heads, which mark the culmination of reduction in the inflorescence, are found in *Adenanthos* (Proteoideae) and *Lambertia uniflora* (Grevilleoideae) and provide good example of parallelism in evolution of the inflorescence in the two sub-families.

In *Euplassa* lateral peduncles as well as pedicels are found (Fig. 22) while in *Kermadecia* and *Gevuina* the flowers are nearly sessile but show lateral peduncles (Fig. 5). The bracts of the individual flowers are usually suppressed; the common bracts are either scaly or membraneous (Figs. 10, 11, 13). The flowers are hermaphrodite, 4-merous and zygomorphic. The perianth is sepaloid or petaloid and polyphyllous; it is antero-posterior in the relatively primitive taxa (*Euplassa, Gevuina*) and diagonal in other genera; a transition is noticed in *Strangea* and *Kermadecia*. The stamens are shortly filamented and apiculate in *Euplassa, Kermadecia, Gevuina* and *Finschia* (Fig. 6); in others they are nearly sessile and non-apiculate. In *Hakea victoriae* and

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FIGS. 1–21. 1–9, *Gevuina* sp. 1, 2, *G. avellana* 1, a branch with inflorescence. ×$\frac{1}{4}$. 2, a leaf from the base of a branch. ×$\frac{1}{6}$. 3, a branch of *G. bleasdalei*. ×$\frac{1}{3}$. 4, a branch of *G. papuana*. ×$\frac{1}{3}$. 5–8, *G. bleasdalei*. 5, a flower pair. ×3. 6, a stamen. ×6. 7, 8, base and top of pistil. ×6. 9, fruit of *G. avellana*. ×1. 10, 11, *Hakea nitida*. 10, an inflorescence. ×2. 11, a flower pair.and common bract. ×3. 12–19, *Strangea* sp. 12–14, *S. linearis*. 12, a branch with inflorescences. ×1. 13, a flower pair. ×3. 14, an open flower. ×4. 15–18, *S. stenocarpoides*. 15, an inflorescence. ×2. 16, a dehisced fruit. ×$\frac{1}{2}$. 17, a seed with testa removed on one side. ×1. 18, an embryo. ×1. 19, an inflorescence of *S. cynanchicarpa*. ×1. 20, 21, *Finschia chloraxantha*. 20, a branch with inflorescence and fruits. ×$\frac{1}{6}$. 21, pistil and nectary. ×3.

H. laurina the anthers are adnate throughout their length with the tepal midribs.

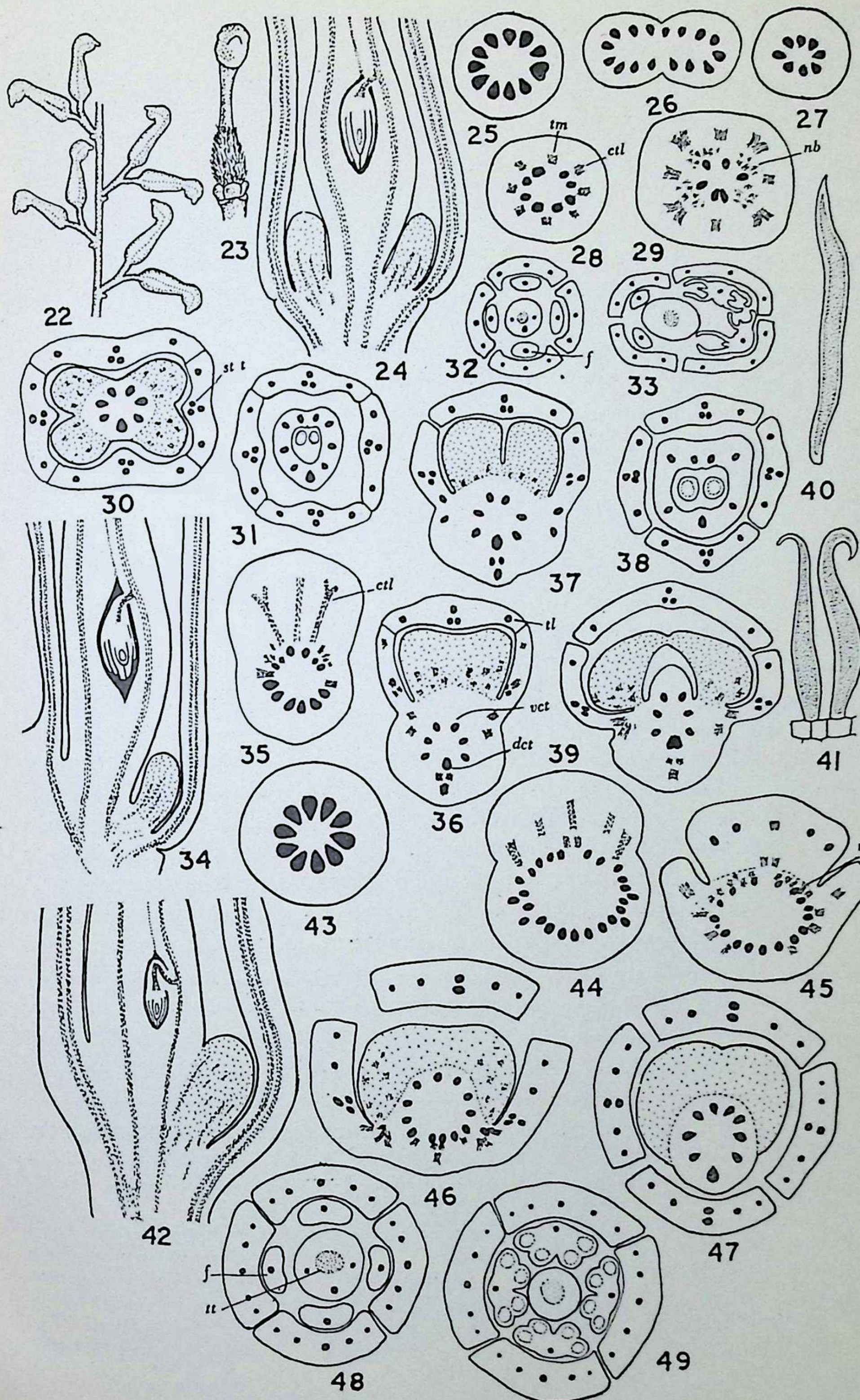
The ovary is either stipitate or sessile, sometimes both conditions being found in the same genus (*Strangea, Grevillea*). Puri (1967) considers the stipitate condition as relatively more primitive. The style-end is club-shaped in *Finschia* and *Kermadecia* and the stigma is devoid of a pollen-collecting apparatus (Figs. 21, 68); in other genera the stigma is lateral or oblique and subtended by a discoid pollen-collecting apparatus (Figs. 8, 14). All stages in the evolution of the pollen-collecting apparatus are noticed within *Grevillea* (Venkata Rao 1966*b*).

The flowers in all genera show a nectary. It consists of 4 separate lobes only in *Euplassa* (Figs. 23, 30), resembling the condition in some Persoonieae. In *Grevillea leucopteris* the lobes are connate into a massive perigynous ring. In other taxa it is incomplete, being 2-lobed in taxa with antero-posterior perianth, viz. *Gevuina, Kermadecia, Strangea stenocarpoides* and *S. cynanchicarpa*, or 3-lobed in those with diagonal perianth (Figs. 7, 21, 55, 60, 64, 65, 70). The nectary may be found either directly on the thalamus (Figs. 24, 34, 61) or adnate to the base of the perianth as in *Hakea crassifolia* or on the stipe or base of the ovary (Figs. 47, 58). In *H. neurophylla* and *Grevillea synaphae* the flowers are glandless due to complete reduction. The wide range of variation (sometimes within a genus like *Grevillea*) shows that it is not a morphological entity but only an enation of the thalamus or other floral organs.

Though the flowers are zygomorphic in all genera, the degree of zygomorphy varies. In *Euplassa* they are structurally regular but become slightly zygomorphic due to the curvature of the tip of flower bud. In other genera they become increasingly zygomorphic due to the structural modifications in one or more floral organs. The highest zygomorphy (in the tribe as well as in the whole family) is noticed in *Grevillea rosemarinifolia* due to oblique thalamus, gibbous perianth, strong curvature of the flower bud, structural modification of tepals of anterior and posterior sectors in size and shape, curved style, lateral stigma with pollen-collecting apparatus and incomplete, cushion-shaped nectary.

The fruit and seed also show much variation. In the relatively primitive taxa (*Euplassa, Gevuina* and *Finschia*) the fruits are rounded nuts (Figs. 9, 20). In *Grevillea* the fruit is a follicle which on dehiscence forms a single

FIGS. 22–49. Floral anatomy. 22–33, *Euplassa cantareirae*. 22, part of inflorescence. ×1. 23, pistil and nectary. ×3. 24, L.S. base of flower. 25, 26, T.S. lateral peduncle. 27, T.S. pedicel. 28–33, T.S. flower bud at various heights. 24–33, ×30. 34–41, *Gevuina avellana*. 34, L.S. base of the flower. ×30. 35–39, T.S. flower bud at different heights. ×25. 40, 41, hairs from tepal and ovary wall respectively. ×80. 42–49, *Kermadecia sinuata*. 42, L.S. base of flower bud. 43, T.S. pedicel. 44–49, T.S. flower bud at various heights. 42–49, ×8. (*tm* = tepal midrib; *ctl* = conjoint tepal lateral; *nb* = nectary bundles; *f* = filament; *tl* = tepal lateral; *vct* = ventral carpellary traces; *dct* = dorsal carpellary trace; *tt* = transmitting tissue; staminal trace.)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FIGS. 22–49.

concave valve. In *Strangea* it is relatively large and dehisces (capsule-like) into two valves which are smooth on the outside. In *Hakea* the fruit is very woody and splits characteristically into two plano-convex valves on the face of each of which one seed is left exposed. The outer surface of the valve may be smooth, warty or tubercular, these features being of taxonomic importance.

The fruits in *Gevuina* and *Finschia* bear a single, large, rounded wingless seed; the embryos are large and rounded with fleshy cotyledons which store abundance of food. Netolitzky (1926) thinks that embryos which store food are relatively more primitive than those which do not. In *Strangea* the fruit bears a single, flat seed with a wing which extends on both micropylar and chalazal sides (Fig. 17). In *Grevillea* and *Hakea* the fruits are 2-seeded; the seeds are flat on the side of mutual contact and convex on the reverse. They are usually winged; the wing is samaroid in *Hakea* and symmetrical all round in *Grevillea*. The embryos in *Strangea*, *Grevillea* and *Hakea* are flat and show foliaceous cotyledons with basal lobes (Fig. 18).

Floral anatomy—The stalk of the flower pair in *Gevuina*, *Kermadecia* and *Euplassa* (morphologically a lateral peduncle) shows a ring of vascular bundles at base which expands laterally coincident with an increase in the number of bundles due to splitting. It becomes 2-lobed and finally splits into two rings, each of which functions as the stele of one flower (Figs. 25–27). The base of the pedicel in the different taxa shows four or more vascular bundles (Figs. 43, 52, 62). The origin and emergence of traces for floral organs occur either symmetrically or asymmetrically according as the thalamus is horizontal (Figs. 24, 28, 29, 50, 53, 54, 57) or oblique (Figs. 34–39, 42, 44–47, 63, 64). Only in *Kermadecia* the tepals (which are relatively massive) are 5-traced (Figs. 46–49); in others they are 3-traced. The tepal marginals in all taxa arise as conjoint traces. So, a whorl of 8 traces arises in all taxa except *Kermadecia* in which 16 traces arise. The nature of the traces varies according to the position of the perianth on the thalamus; in taxa with antero-posterior perianth, the traces given off along the antero-posterior and lateral radii function as the tepal midribs and the diagonal ones as the conjoint marginals while in those with diagonal perianth, the diagonal traces are the tepal midribs and the alternate ones the conjoint tepal laterals. In all taxa the conjoint

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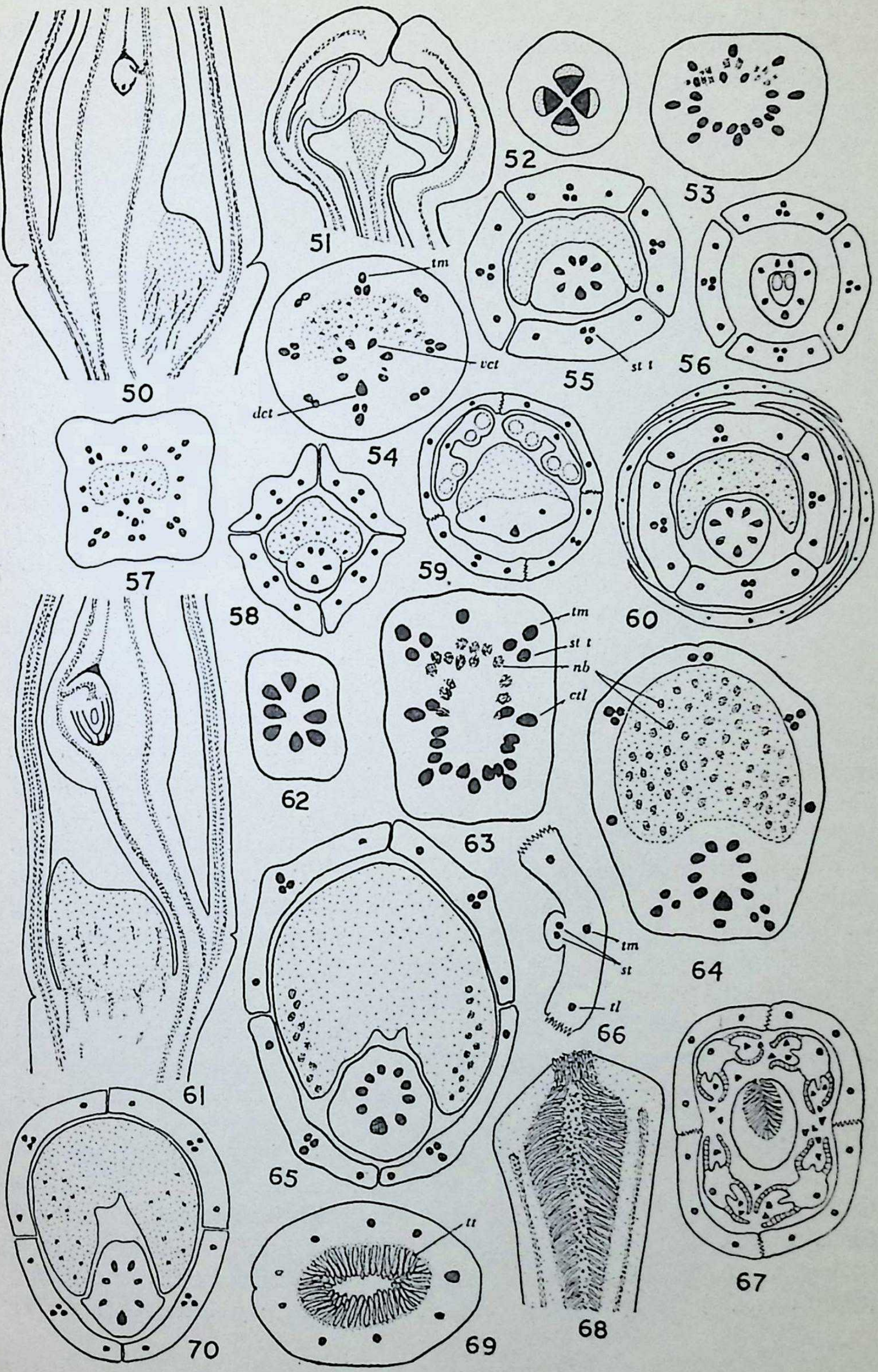
FIGS. 50–70. Floral anatomy. 50–60, *Strangea* sp. 50–56, *S. stenocarpoides*. 50, L.S. base of flower bud. ×35. 51, L.S. top of flower bud. ×25. 52, T.S. pedicel. 53–56, T.S. flower bud at different heights. 52–56, ×30. 57–59, T.S. flower bud of *S. linearis*. ×30. 60, T.S. flower bud of *S. cynanchicarpa* together with the involucral bracts. ×25. 61–69, *Finschia chloraxantha*. 61, L.S. base of flower bud. ×25. 62, T.S. pedicel. 63–65, 67, T.S. flower bud at different heights. ×30. 66, T.S. tepal and the associated stamen. ×25. 68, L.S. stigma. ×18. 69, T.S. style. ×25. 70, T.S. flower bud of *Finschia ferruginea*. ×20. (*tm* = tepal midrib; *tl* = tepal lateral; *st t* = staminal trace; *nb*; = nectary bundles; *ctl* = conjoint tepal laterals; *vct* = ventral carpellary traces; *dct* = dorsal carpellary trace; *tt* = transmitting tissue; *st* = stamen.)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FIGS. 50–70.

laterals split radially close to their origin and demarcate the marginal bundles of adjacent tepals (Figs. 29, 35, 46, 54).

The staminal traces in all genera are organized as pairs of bundles which are given off along the tepal midrib radii and from the same gaps of floral stele; these traverse parallel to the tepal midribs in the limbs of tepals. The two bundles of each trace may fuse together either at the base of tepal or higher up or even just before entering the filament (Figs. 31, 46, 47, 55, 56, 59, 66). The lower the level at which the fusion occurs, the more evolved the condition is to be considered. Only in a few species of *Hakea* (*H. laurina*, *H. victoriae*) the staminal trace and tepal midrib arise as common traces. These split tangentially higher up and demarcate the respective bundles. Since in these species the anther is adnate with the tepal midrib the two bundles traverse parallel to each other in the tissue common between the tepal and stamen. This condition represents the maximum adnation between the tepal and stamen noticed in Grevilleeae. The staminal bundle extends into the appendage of the connective where it is present. The stamens in *Euplassa*, *Kermadecia*, *Strangea stenocarpoides* and *Finschia* sp. show short flat filaments (Figs. 32, 33, 48, 51), a condition which should be regarded more primitive than the nearly sessile condition of the anthers in *Grevillea* and *Hakea*.

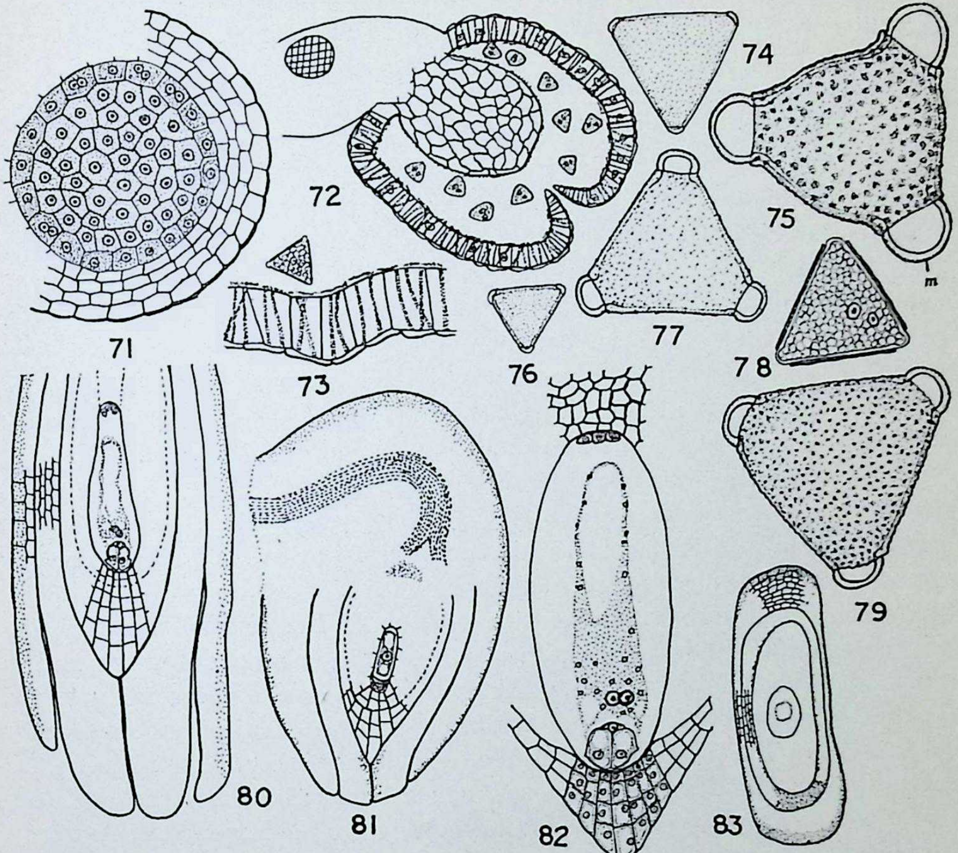
After the emergence of tepal-stamen supply, the bundles of the floral stele abstrict some strands for the nectary, bend inwards and function as the carpel traces. Where the nectary is symmetrical, the nectary strands are cut off either all round the floral stele as in *Grevillea leucopteris* or from stelar bundles of four alternitepalous sectors as in *Euplassa* (Figs. 29, 30). In flowers with incomplete, 2- or 3-lobed nectaries they are derived from stelar bundles of the respective sectors (Figs. 54, 57, 58, 63, 64, 70). Usually the nectary bundles branch and terminate at about half the height of the nectary.

The carpels in all genera are five or more traced; 3-traced carpels are not noticed in Grevilleeae. Three or more bundles extend into the style and traverse nearly to the stigma (Figs. 51, 59, 68, 69). The core of the style shows elongated glandular cells of transmitting tissue. Usually the style is solid (Figs. 48, 49) but in *Finschia* it shows a stylar canal which is lined by finger-shaped, 1-celled hairs (Fig. 69). These run continuously with similar hairs of the stigmatic region (Figs. 67, 68).

The floral organs show rust-coloured, 1-celled epidermal hairs (Figs. 40, 41).

Microsporangium, microsporogenesis and male gametophyte

The anther wall comprises the epidermis, well-developed fibrous endothecium, two middle layers and the tapetum which is of the glandular type (Figs. 71–73). There is a stomium at which the anther dehisces longitudinally. In *Strangea*, *Finschia*, *Kermadecia* and some species of *Grevillea* (e.g. *G. vestita*) the sporogenous cells show a secondary increase (Fig. 71) while in *Hakea* and some other species of *Grevillea* like *G. rosemarinifolia* they function directly as the sporocytes. In the latter case, only a few large pollen grains are produced per anther loculus while in others they are more numerous and small in size. The microspore tetrads are tetrahedral and cytokinesis of the sporocyte is brought about by simultaneous furrowing.



FIGS. 71–83. 71–79, microsporogenesis and male gametophyte. 71, T.S. young anther loculus of *Strangea stenocarpoides*. ×120. 72, T.S. dehisced anther lobe of *Finschia chloraxantha*. ×55. 73, part of mature anther wall and a pollen grain of *Kermadecia sinuata*. ×120. 74–79, pollen grains. ×800. 74, *Gevuina bleasdalei*; 75, *Grevillea hilleana*; 76, *Strangea cynanchicarpa*; 77, *Kermadecia sinuata*; 78, *Euplassa cantareirae*; 79, *Finschia chloraxantha*. 80, L.S. micropylar part of ovule of *Kermadecia sinuata*. ×120. 81–83, *Finschia chloraxantha*. 81, L.S. ovule with 1-nucleate embryo sac. ×75. 82, L.S. part of nucellus with embryo sac. ×200. 83, T.S. ovule. ×50. (*m* = membrane.)

The pollen grains in all genera are triangular, triporate, angulaperturate and 2-celled at the shedding stage (Figs. 74–79). Starch is accumulated in the cytoplasm of the pollen grain. The intine is as thick as the exine or thinner and protrudes slightly or markedly through the germ pore. In species of

Grevillea and *Hakea* in which the germ pores are wide, it forms knob-like swellings which are covered over by special protective membranes (Fig. 75). The ornamentation of the exine ranges from granular to papillate and spinescent patterns. In their large size and complex exine ornamentation, the pollen grains of *Grevillea* and *Hakea* conform to the most highly evolved type in Proteaceae. The pollen grains of *Grevillea vestita* are unique in being polymorphic; they show 3–8 germ pores according to which they vary from triangular to wheel-shaped structures (Venkata Rao 1964).

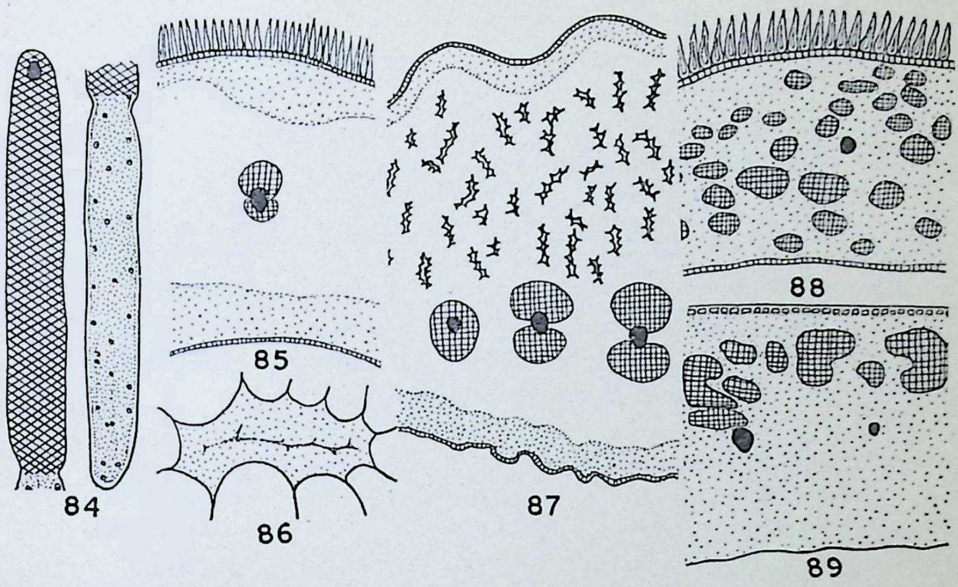
Megasporangium, megasporogenesis and female gametophyte

The ovules in all genera are bitegmic and crassinucellate. In *Euplassa, Gevuina, Strangea* and *Kermadecia* they are orthotropous and pendulous while in *Finschia* and most species of *Grevillea* and *Hakea* they are hemianatropous and lateral. In a few species of *Grevillea* (e.g. *G. trifida*) they are anatropous and basal, which seems to be the most highly advanced condition in the family. The funicular vascular bundle branches and forms a ring of strands in the chalaza (Fig. 81). The outer integument is biseriate and the inner usually 3–4 cells thick. In *Finschia chloraxantha* the outer integument is 8–10 cells thick at the margins and thinner elsewhere (Fig. 83). Only the inner integument forms the micropyle (Figs. 80, 81). The parietal tissue consists partly of the derivatives of the primary parietal cell and partly of the nucellar cap (Figs. 80, 81). A hypostase of thick-walled cells is usually found below the embryo sac (Fig. 82).

The female archesporium consists of a single hypodermal cell. In *Grevillea rosemarinifolia* it is multicellular and several embryo sacs are formed in an ovule (Venkata Rao 1966*a*). The embryo sac develops according to the Polygonum type (Figs. 81, 82). A filiform apparatus is noticed in some species of *Grevillea*. The two polar nuclei fuse only at the time of fertilization. The antipodals are usually inconspicuous and ephemeral; in some species of *Grevillea* they are seen to persist till the early stages of endosperm development. Starch grains are noticed in the embryo sac of some species of *Grevillea* and *Finschia chloraxantha* (Fig. 82).

Endosperm—Fertilization is porogamous. The endosperm is of the Nuclear type. The endosperm nuclei formed after the first free nuclear divisions become distributed throughout the embryo sac. The micropylar part then becomes cellular while the rest of the endosperm remains free nuclear and functions as the haustorium. In *Strangea cynanchicarpa* the endosperm haustorium is of a simple type and does not show either aggressive growth or secondary haustorial lobes (Fig. 84); it resembles the haustorium in *Orites revoluta* and *Knightia excelsa* (Venkata Rao 1967). Complex haustoria which show aggressive growth and secondary haustorial lobes are seen in *Grevillea* and *Hakea* (Venkata Rao 1966*b*, 1968*a*). Mature seeds are non-endospermic.

Pericarp—In *Grevillea* and *Hakea* sclerenchyma is noticed both in association with the vascular bundles and as additional strands distributed either near to the outer surface of the pericarp or throughout its thickness (Figs. 88, 89). The epidermal hairs of the ovary wall are persistent in some taxa. Eventually all the parenchyma become thick-walled and lignified and the pericarp becomes very woody.



FIGS. 84–89. 84–86, *Strangea cynanchicarpa*. 84, endosperm. ×12. 85, T.S. young pericarp. ×30. 86, a thick-walled 'cell' from old pericarp. ×85. 87, T.S. pericarp of *S. stenocarpoides*. ×13. 88, T.S. pericarp of *Grevillea acanthifolia*. ×25. 89, T.S. pericarp of *G. endlicheriana*. ×30.

Thick-walled cells of a special type are noticed in *Strangea*. In the young fruit sclerenchyma are noticed only in association with the vascular bundles; the remaining pericarp consists of thin-walled parenchyma with cellulose walls (Fig. 85). As the fruit grows, cell wall material (which stains lightly with safranin) becomes deposited on the parts of cell walls abutting on an intercellular space. Such 'cells' are usually described as tubular collenchyma (Fig. 86). These are 4- or 5-sided to start with; as more and more cell wall material becomes deposited, the adjacent cells become wedged apart and the intercellular space extends. The material continues to be deposited on the walls of the newly exposed cells. Thus these 'cells' grow and become caterpillar-like as the pericarp becomes older (Figs. 86, 87). Such thick-walled cells are noticed characteristically in all the species of *Strangea* but are not seen in any other genus of Proteaceae studied.

DISCUSSION

From the account given above, we can not only trace the evolutionary trends within the Grevilleeae but formulate the characteristics of the ancestral stock. The ancestral Grevilleeae seem to have been large rain-forest trees with large, compound, mesophytic leaves; large terminal paniculate inflorescences which were sometimes cauliflorous; small nearly regular flowers; large indehiscent nuts; large wingless seeds, and rounded fleshy embryos. The relatively primitive genera of the tribe (*Euplassa*, *Gevuina*) resemble closely the Persoonieae suggesting their origin from this tribe. In several features the two genera, *Grevillea* and *Hakea*, mark the culmination of the evolutionary tendencies not only within Grevilleeae but in the whole of Proteaceae. The following trends of evolution are evident within the tribe:

TABLE II

No.	Primitive	Evolved
1	Large trees of rain-forests	Shrubs and undershrubs of exposed spaces
2	Large, bicompound or compound, mesophytic leaves	Small, simple, acicular or spinescent, xerophytic leaves
3	Diffuse, terminal panicles with more than two flowers on lateral branches	Uniaxial pseudoracemes with paired flowers, or small few-flowered corymbs or 1-flowered involucrate heads
4	Flowers inconspicuous, nearly regular with sepaloid, antero-posterior perianth	Flowers strongly zygomorphic with petaloid, diagonal perianth
5	Stamens with short free part of filaments and produced connectives	Stamens nearly sessile, not appendaged
6	Ovary stipitate; stigma simple, terminal	Ovary sessile; stigma lateral, subtended by pollen-collecting apparatus
7	Nectary symmetrical, either 4-lobed or ring-like	Nectary zygomorphic, incomplete, 2- or 3-lobed
8	Fruit indehiscent, globose nut	Fruit flattened, follicle or 'capsule'
9	Embryo rounded with thick, fleshy cotyledons	Embryo flat with thin foliaceous cotyledons
10	Floral organs and traces free	Floral organs and traces showing connation and adnation
11	Pollen grains small, numerous with smooth exine	Pollen grains large, few per loculus, exine intricately ornamented
12	Ovules orthotropous, pendulous from the top of loculus	Ovules hemianatropous and lateral or anatropous and basal.

The cytological evidence corroborates the conclusion drawn on morphological grounds that Persoonieae are the nearest to the ancestral stock of Grevilleeae. The Persoonieae contain, in addition to some polyploids, the two diploid genera, *Bellendena* ($n = 5$) and *Persoonia* ($n = 7$); both 5 and 7 seem to be the basic numbers in the family though $n = 14$ is found more commonly. In Grevilleeae, $n = 14$ is found in the relatively primitive genus *Gevuina* (the chromosome number in *Euplassa* is not known); $n = 11$ is found in the relatively more advanced *Strangea* and $n = 10$ (which is the smallest haploid chromosome number in the Proteaceae) in the two most highly evolved genera, *Grevillea* and *Hakea*. Morphological evolution, therefore, seems to have been accompanied by a reduction in chromosome numbers.

The distribution pattern of the genera of Grevilleeae throws some light on the probable centre of origin of the tribe. *Hakea* and *Strangea* are wholly endemic to Australia being distributed in East and West Australia; *Euplassa* is wholly endemic to S. America. *Grevillea*, with the major number of species centered in Australia, extends to the neighbouring islands New Guinea and New Caledonia. *Gevuina* is common to East Australia, New Guinea and S. America. *Kermadecia* and *Finschia* are not Australian but are found in Pacific islands. Out of the 7 genera, therefore, four are found in E. Australia, three in W. Australia, two in S. America and two in the islands off east coast of Australia. E. Australia shows community at the generic level with the neighbouring islands as well as with far off S. America, which W. Australia does not. So, E. Australian region on a once connected land mass (Gondwanaland) seems to be the probable centre of origin for the tribe. The Persoonieae from which the Grevilleeae are supposed to have originated are also better represented in this region than elsewhere. The present-day disjunction of the tribe seems to have been brought about due to continental drift. When S. America separated from the common land mass, it seems to have received the primitive stock.

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EMBRYOLOGICAL INVESTIGATIONS ON *BIXA ORELLANA* LINN.*

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(Communicated by B. M. Johri, F.N.I.)

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Bixa orellana alone constitutes the family Bixaceae. The endosperm is of the Nuclear type. Cell formation starts from the micropylar end. The reserve food in the endosperm is starch. The embryogeny does not conform to any set type. After fertilization there is incurving of integuments in the chalazal region. The outermost layer of the inner integument forms the palisade-like layer and the cells of the inner hypodermal layer become hourglass-shaped. In a mature seed the outer integument is soft and full of dye cells, whereas the inner integument is multilayered and hard. The available embryological data show that Bixaceae have affinities with Cistaceae and Cochlospermaceae and almost none with Flacourtiaceae, Frankeniaceae, Tamaricaceae and Tiliaceae.

Introduction

Bixa orellana Linn. is a monotypic genus of the family Bixaceae. In India *Bixa* is grown mostly in the South as an ornamental and a hedge plant. Its mature seeds yield a red dye known as '*Arnotto*', which was previously used for colouring cotton and silk. Since the dye is not fast, it is at present employed for colouring edible materials, ointments, polishes, hair oils, etc.

The family Bixaceae has been put in the order Parietales by some taxonomists (Lawrence 1951; Rendle 1952); others assign it to the order Bixales (Johansen 1950; Hutchinson 1959) or to the Violales (Engler 1964). Johansen employed embryological characters, while Hutchinson based his grouping mainly on external morphology. Engler (1964) felt that if the Guttiferales are removed from the Parietales, the remaining taxon, i.e. Violales, forms quite a homogeneous and natural group.

This investigation was undertaken because there is no detailed work on the embryology of Bixaceae. On the basis of the present findings, a comparison of the Bixaceae with the allied families has been made.

Material and Methods

The material of *Bixa* was collected from the Indian Agricultural Research Institute, New Delhi, during October–February, 1964–65. Formalin-acetic-

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* This work is part of a Ph.D. thesis approved by the University of Delhi in 1967.

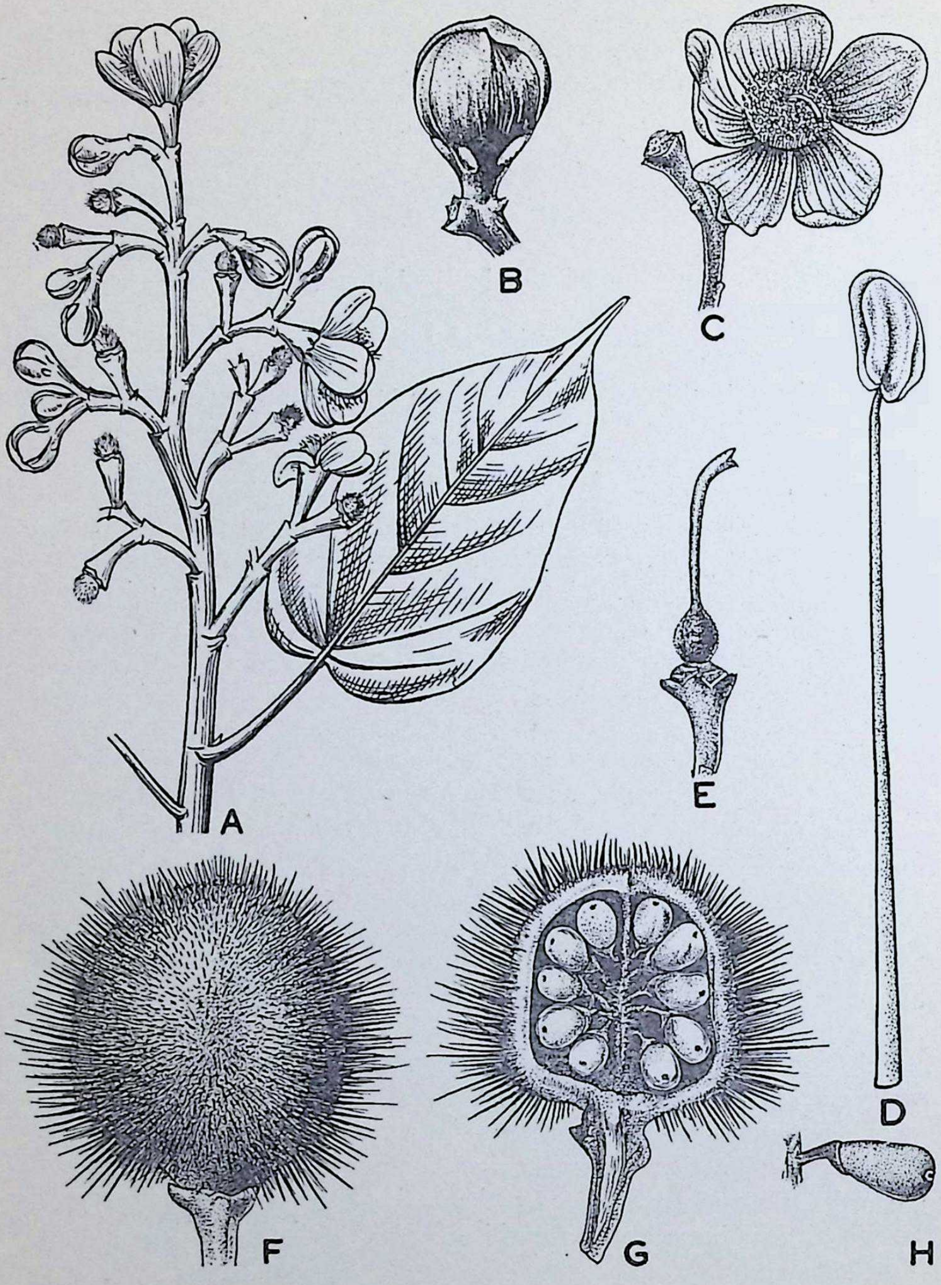

Fig. 1 A–H. *Bixa orellana*, floral morphology. A, twig with inflorescence. × 34. B, bud showing gland-like structures at the base of sepals. × 1·0. C, flower. × 3·4. D, stamen. × 5·9. E–F, young and mature fruits. × 68. G, one fruit valve showing the placenta bearing two rows of seeds. × 68. H, mature seed with a collar-like outgrowth at the micropylar end. The white, circular zone at the chalazal end represents thickened area. × 73.

alcohol was used for fixation and 70 per cent alcohol for preserving the material. Mature fruits were also fixed in Nawaschin's modified solution (Johansen 1940). Dehydration and embedding were done in the usual way. Sections were cut between 6 μ and 32 μ. Hard seeds were soaked in glycerine-alcohol mixture (30 cc of pure glycerine, 20 cc of 50 per cent alcohol), after embedding, for 15 days or so. Safranin-fast green combination was used for staining.

OBSERVATIONS

Floral morphology

Bixa orellana has two forms: one with white flowers and green capsules, and the other with pink flowers and brownish-red capsules. The following description is based only on the latter. The inflorescence is a panicle (Fig. 1 A). The buds are somewhat asymmetrical (Fig. 1 B). The flowers (Fig. 1 C) are bisexual, regular and hypogynous. There are usually five concave, distinct, imbricate sepals with gland-like structures at their base. The sepals fall off as soon as the bud opens. The five petals are obovate, distinct, imbricate and dotted (the dots representing the dye cells). The numerous free stamens develop centrifugally. The anthers are basifixed, dithecous and horseshoe-shaped (Fig. 1 D). The ventral arm of the anther (i.e. the one with filament) is shorter than the dorsal one. The filaments are long and extremely thin (Fig. 1 D). There is a single vascular trace to the anther (Fig. 2 A, B) which terminates in a small hook.

The gynoecium is bicarpellary and syncarpous. The ovary is unilocular with two parietal placentae bearing numerous ovules. The fruit is a loculicidal, echinate, two-valved, globose capsule. The soft and pointed outgrowths on the ovary wall enlarge and become stiff at maturity (Fig. 1 E–G). The number of ovules per ovary may be 50 or more, but all of them do not develop further. The mature seeds show a cap-like, funicular outgrowth (Fig. 1 H) and are covered with red dye.

Some abnormal anthers have also been observed (*see* Fig. 2 C–H).

Microsporangium, microsporogenesis and male gametophyte

Serial transections of anther reveal different configurations (Venkatesh 1956). In the middle the thecae of both the arms are united and hence eight microsporangia are seen. Most of the diagrams have been made from this region. A transection of a young anther (Fig. 3 A) shows a single hypodermal archesporial cell (Fig. 3 B) in each of the eight lobes. The archesporial cells undergo periclinal division. The layer of parietal cells gives rise to three or four wall layers, the innermost of which functions as the tapetum (Fig. 3 C, D). The sporogenous cells divide repeatedly and ultimately form the microspore mother cells (Fig. 3 E, F).

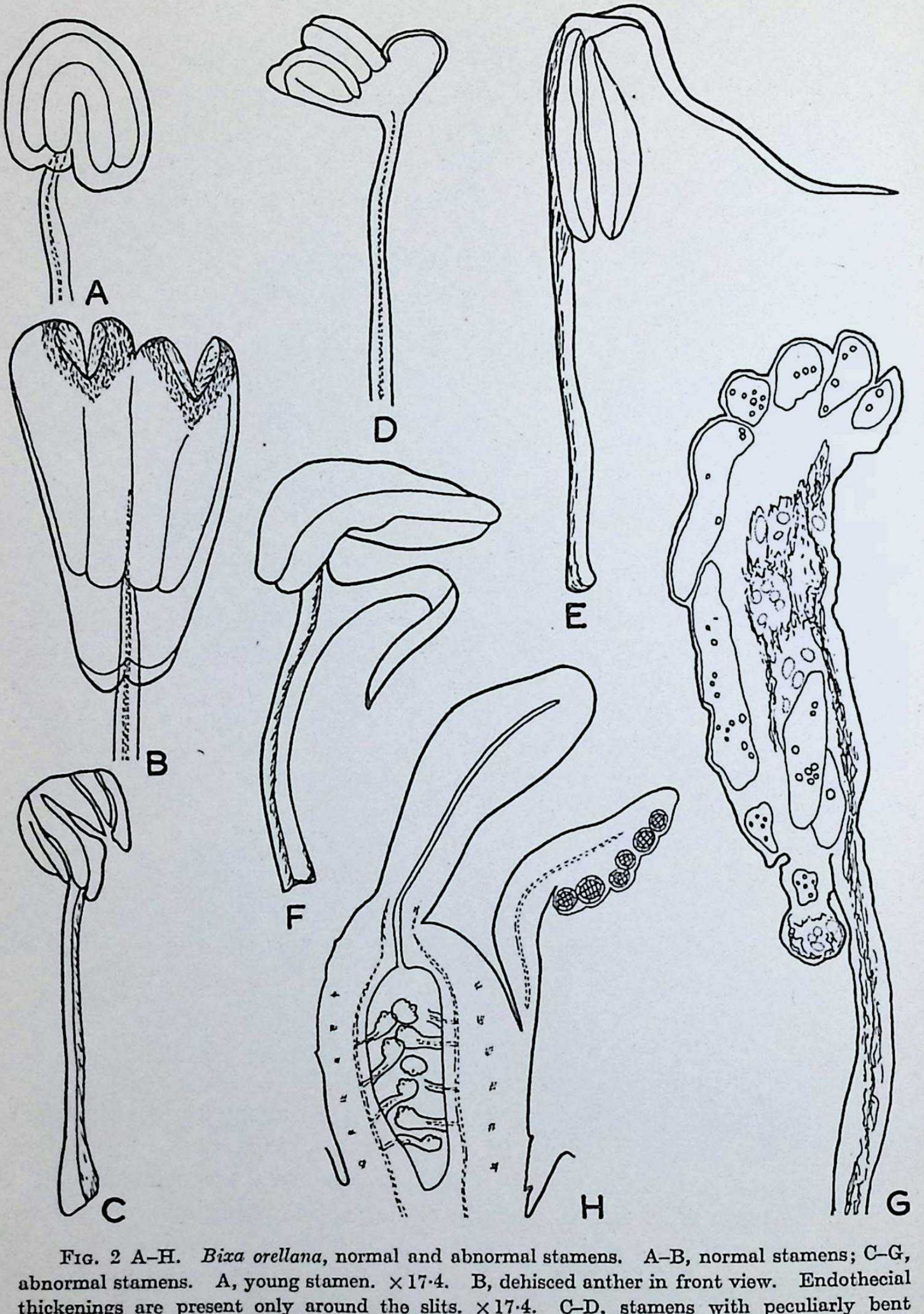

FIG. 2 A–H. *Bixa orellana*, normal and abnormal stamens. A–B, normal stamens; C–G, abnormal stamens. A, young stamen. ×17·4. B, dehisced anther in front view. Endothecial thickenings are present only around the slits. ×17·4. C–D, stamens with peculiarly bent thecae. ×4·4. E–F, thick and large stamens with a sterile appendage. ×4·4. G, l.s. stamen with several microsporangia. The vascular trace extends almost to its tip. ×17·4. H, l.s. carpel with a stamen arising from the ovary wall. ×13.

In a mature anther the epidermal cells become papillate (Fig. 3 I, J). Dehiscence takes place by two slits at the bend of the thecae and endothecial thickenings are confined around this area (Fig. 3 I, K). The thickenings develop as radial bands (Fig. 3 K) which are often fused towards the inner tangential walls of the cells (Fig. 3 J). The middle layers elongate (Fig. 3 G, H), get compressed and finally disappear at the time pollen grains are shed. When the sporogenous cells are multiplying the tapetum becomes 2-layered at places, but its cells are mostly uninucleate (Fig. 3 D, L). During meiotic divisions in the microspore mother cells the tapetal cells enlarge and become binucleate. Their inner tangential and radial walls get covered with 'Ubisch' granules (Fig. 3 H, L), and frequently the cytoplasm shows striations (Fig. 3 H). The tapetum is of the secretory type.

Mucilage appears between the cytoplasm and the cell wall of mother cells at the prophase. Several darkly stained bodies often appear in the cytoplasm. However, their number decreases in later stages (Fig. 3 M–Q). At metaphase II the spindles may be arranged side by side (Fig. 3 Q) or crosswise. At the end of meiosis II some nucleus-like structures are visible (Fig. 3 R, S), which are probably derived from laggard chromosomes (Fig. 3 O). Wall formation is the simultaneous type. Tetrads are mostly tetrahedral (Fig. 3 T), sometimes decussate. Young pollen grains are vacuolated (Fig. 3 U). The vegetative nucleus is often exceptionally large (Fig. 3 V). The pollen grains are tricolporate and are shed at the 2- or 3-celled stage. Pollen grains with as many as five nuclei have been observed (Fig. 3 W–Z).

Megasporangium

The ovules are bitegminal, anatropous and crassinucellar. As the ovular primordia elongate the integuments start differentiating. The outer integument overgrows the inner and both of them form the micropyle, which becomes narrow and zigzag. The derivatives of the nucellar epidermis form a cap, which remains distinct for some time.

Megasporogenesis and female gametophyte

Generally only one archesporial cell differentiates in an ovule (Fig. 4 A). The sporogenous cell enlarges and functions as the megaspore mother cell (Fig. 4 B). At times two or more sporogenous cells are present. They may lie one above the other, diagonally or side by side. In a twin of mother cells (Fig. 4 C) one may divide earlier or both may divide simultaneously to form twin tetrads (Fig. 4 F). The tetrads are usually linear, at times T-shaped. Generally it is the chalazal megaspore which functions (Fig. 4 E), but in some preparations the micropylar megaspore had also enlarged (Fig. 4 D, E), and in still others both the chalazal and the epichalazal megaspores had developed up to the 2-nucleate embryo sac stage (Fig. 4 G).

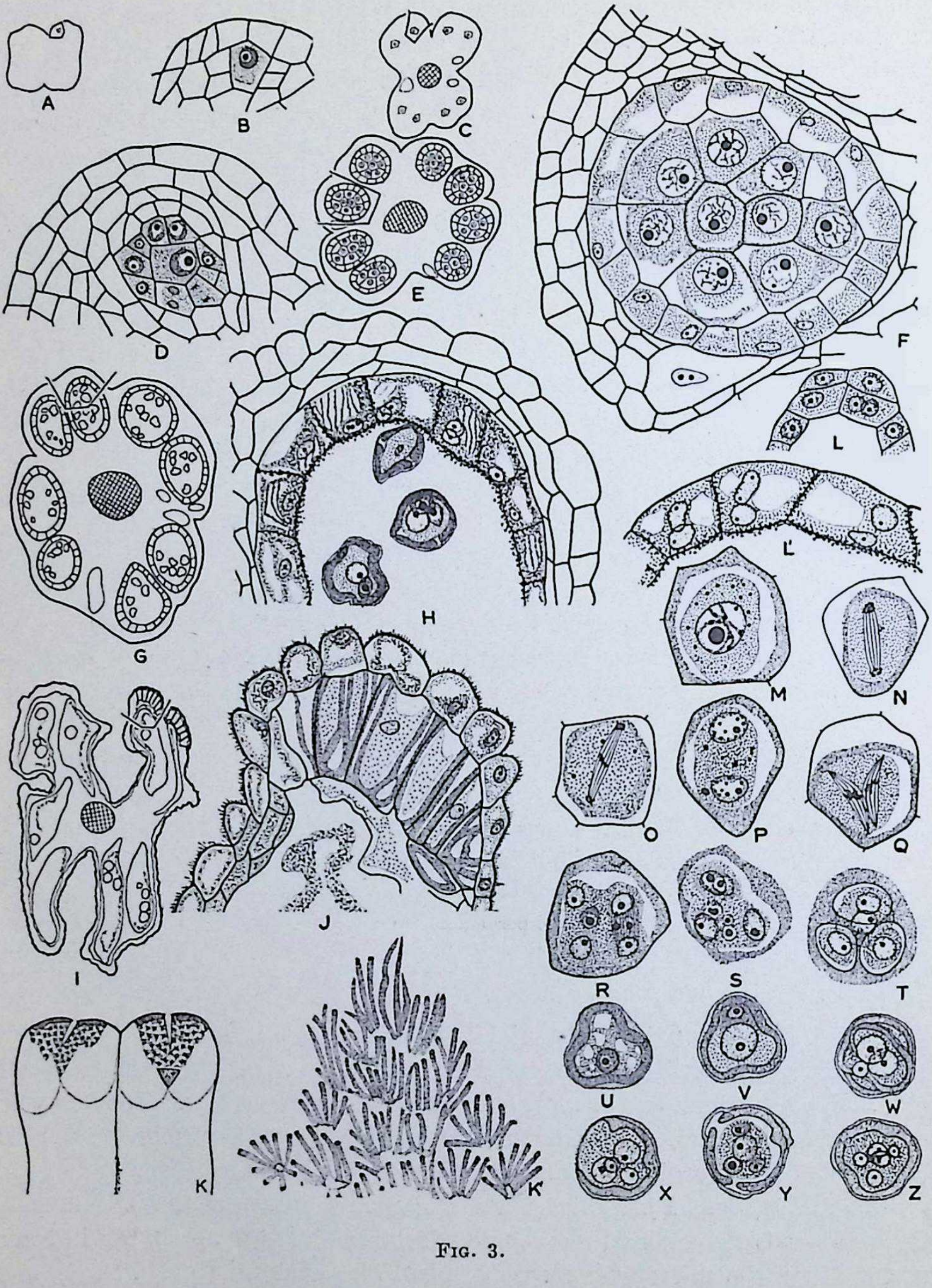
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FIG. 3.

The nucleus of the functional megaspore divides thrice to form the Polygonum type of embryo sac (Fig. 4 H, I). Some embryo sacs with extra pairs of polar nuclei or extra cells at the micropylar end have also been observed. The embryo sac increases greatly in size after its organization (cf. Fig. 4 I, J). The egg may be apical or lateral in attachment, and contains a number of starch grains. The synergids are hooked, have basal vacuoles and often show filiform apparatus. One of the synergids collapses after fertilization, while the other enlarges and persists till the early proembryo stages. It resembles the zygote and its products, and can, therefore, be mistaken as a component of the proembryo. The antipodal cells usually degenerate before the entry of pollen tube. The polar nuclei come to lie in the middle of the embryo sac (Fig. 4 J).

Pollination and fertilization

At the time of pollination the two stigmatic flaps are wide open and copious liquid is secreted in which the pollen grains germinate. Fertilization is porogamous and syngamy is completed later than triple fusion. The pollen tubes persist till the massive nuclear endosperm stage.

Endosperm

The endosperm is of the Nuclear type (Fig. 5 A–D). The nuclei are at first evenly distributed in the peripheral cytoplasmic lining, but later more nuclei aggregate towards the micropylar end. The early nuclear divisions are usually synchronous, but afterwards they are not so (Fig. 5 E). In one preparation the endosperm showed considerable variation in nuclear size (Fig. 5 F).

After hundreds of nuclei are formed the cytoplasm becomes vacuolated and cell formation proceeds from the micropylar end downward (Fig. 5 G, H).

FIG. 3 A–Z. *Bixa orellana*, microsporangium, microsporogenesis and male gametophyte. A, outline of a young anther in cross-section. ×91. B, portion marked in A enlarged to show the archesporial cell. ×637. C, cross-section of slightly older anther. ×91. D, enlarged view of the portion marked in C to show the wall layers and differentiating tapetum. ×637. E, cross-section of anther at the microspore mother cell stage. ×75. F, microsporangium enlarged from E. ×487. G, cross-section of anther with young pollen grains. ×75. H, magnified view of the portion marked in G. The tapetal cells show cytoplasmic striations and 'Ubisch' granules. ×487. I, section of anther at the time of dehiscence. ×88·4. J, enlarged view of the portion marked in I. ×378. K, upper portion of a mature anther showing the extent of endothecial thickenings (diagrammatic). ×40·9. K′, portion marked in K enlarged to show the pattern of thickenings. ×378. L, portion of tapetum at the sporogenous cell stage. ×637. L′, binucleate tapetal cells at the young pollen grain stage. 'Ubisch' granules are present on the inner tangential and radial walls. ×637. M–Q, microspore mother cells at different stages of meiosis. In O laggard chromosomes are seen. ×637. R–S, microspore mother cell after the completion of meiosis. Some additional nucleus-like bodies have also been organized. ×637. T, tetrahedral tetrad. ×637. U–V, uni- and binucleate pollen grains. ×637. W–Z, abnormal pollen grains with supernumerary nuclei. ×637.

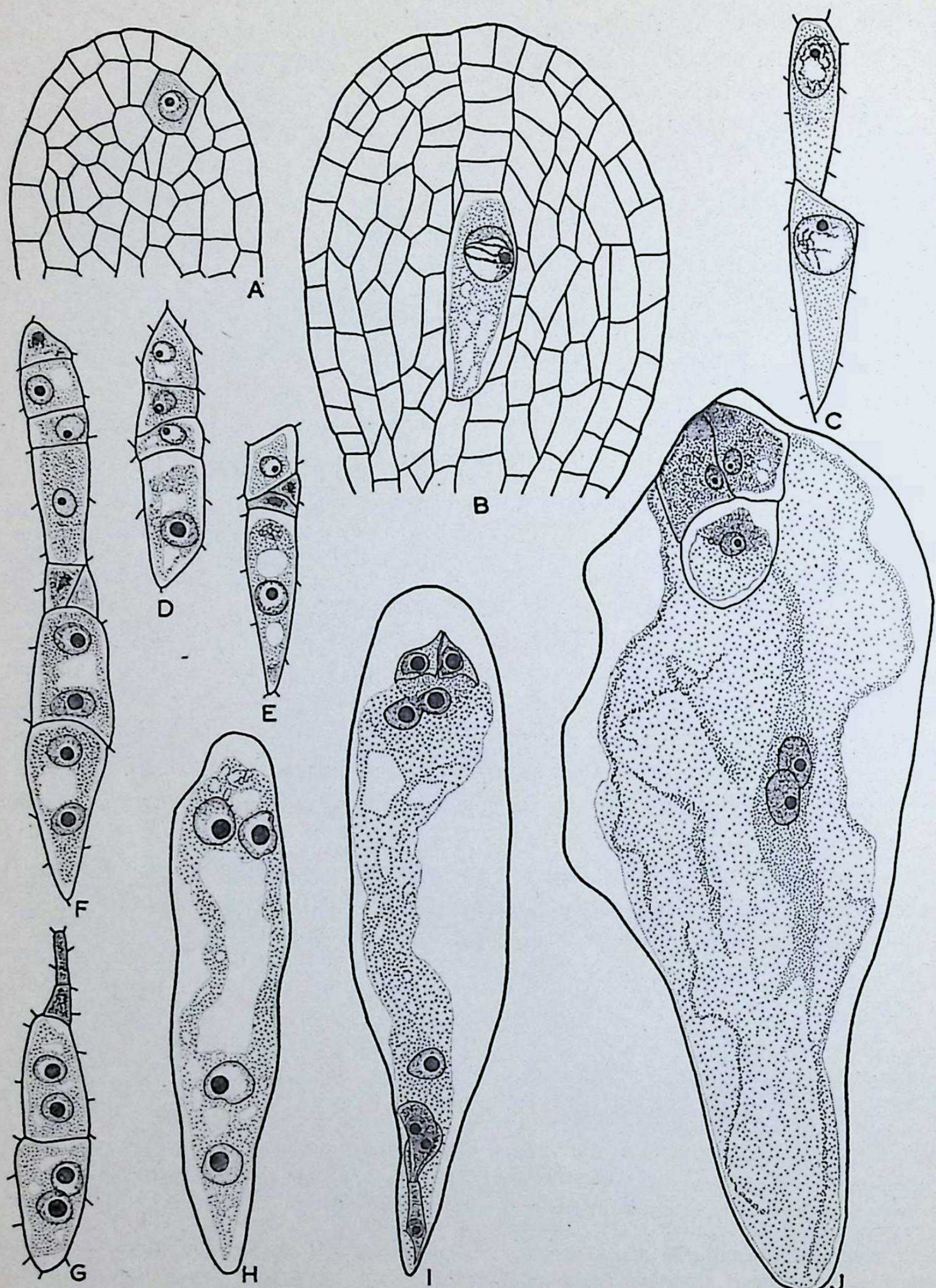
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FIG. 4.

Divisions in the first formed cells result in a massive tissue (Fig. 5 I), which eventually fills the entire central space. The outer one or two layers of endosperm comprise small cells (Fig. 5 J), and excepting these all others store starch at maturity (Fig. 5 K).

Embryo

The zygote undergoes a period of rest which lasts till a large number of endosperm nuclei have been formed. It then divides transversely to form two cells (Fig. 6 A, B). In a subterminally or laterally attached zygote the first division can be mistaken for a vertical one. Both the cells of the two-celled proembryo divide vertically (Fig. 6 C–G). Further divisions in the four-celled proembryo do not follow any set pattern and result in a globular mass in which a few cells are at times larger than the rest (Fig. 6 H–M). Further differentiation of the globular embryo (Fig. 6 N, O) is quite normal (Fig. 6 P–R).

Development of seed

At the megaspore mother cell stage both the integuments comprise 3–4 layers. The cells of the inner integument are densely cytoplasmic whereas those of the outer are vacuolated. By this time, dye cells* start developing in the middle layer(s) of the outer integument.

The post-fertilization changes are as follows:

Funicular base.—A circular outgrowth (*fo*) of parenchymatous cells arises from the funiculus (Fig. 7 B, C) and it looks like a small cap at the tip of seed (Fig. 1 H).

Chalaza.—At maturity the chalaza shows a depressed circular band (16–20 cells in width) of thick-walled cells (*thc*) around the central, tannin-filled raised area (Figs. 1 H; 7 H). The chalazal tissue (*ct*) below the embryo sac (*tes*) shows different zones.

Nucellus.—The cells above and below the embryo sac become slightly thick-walled and constitute the epistase and hypostase (*h*) respectively. The

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* Dye cells occur in all the floral parts.

FIG. 4 A–J. *Bixa orellana*, megasporogenesis and female gametophyte. A, l.s. upper part of ovular primordium with an archesporial cell. ×668. B, l.s. nucellus with megaspore mother cell. Some cells of the nucellar epidermis have divided periclinally. ×668. C, twin megaspore mother cells. ×668. D, linear tetrad with enlarged chalazal megaspore. ×668. E, same, with two degenerated megaspores. ×668. F, twin tetrads. The lower tetrad is T-shaped and two of its megaspores have formed 2-nucleate embryo sacs. ×668. G, linear tetrad; the two lower megaspores have enlarged and become binucleate. ×668. H, four-nucleate embryo sac. ×668. I, organized embryo sac. The antipodal cells have dense contents. ×668. J, same, at the time of fertilization. The antipodal cells have degenerated and disappeared. The synergids show filiform apparatus and the egg contains starch grains. ×511.

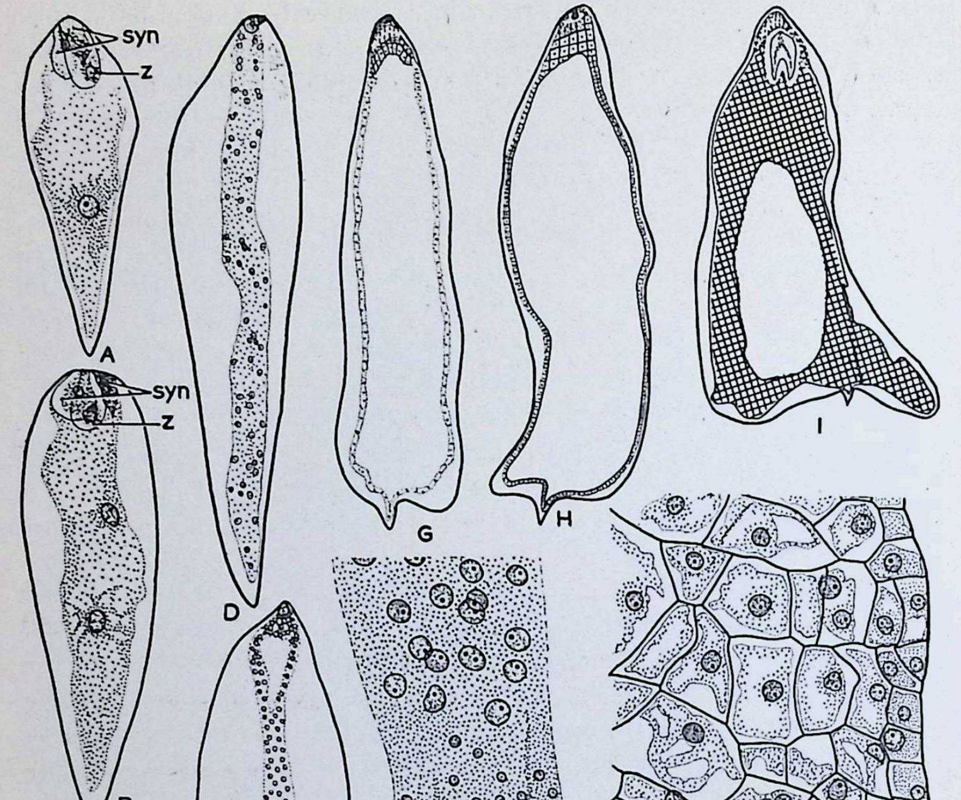
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FIG. 5.

cells of nucellar epidermis in the chalazal region elongate radially and undergo repeated anticlinal divisions. Later, periclinal divisions also occur and generally it becomes difficult to distinguish their derivatives (*dne*; Fig. 7 D–G). In the developing seed, remnants of some nucellar cells (*mu*) are seen at the tip, but ultimately only a few nucellar cells (*pnc*) other than those of the hypostase persist (Fig. 7 H).

Outer integument.—While the outer epidermis (*oloi*) does not alter much, more and more of dye cells (*dc*) differentiate in the middle layers. The dye cells become multinucleate (Fig. 8 A–F) and vacuolated. The nuclei at times show fusions. The vacuoles coalesce and the nuclei are pushed to the peripheral layer of cytoplasm (Fig. 8 G). In a mature seed almost all the cells of middle layers are converted into dye cells. Finally, the nuclei degenerate and the cells become filled with the dye* (Fig. 8 H).

The cells of the inner epidermis in the chalazal region (*iloi*) elongate and get obliquely oriented. At the early globular embryo stage these cells assume a S-shape configuration along with the palisade-like layer of the inner integument (Fig. 7 E–H).

Inner integument.—During the early stages of embryo development the integument becomes 7–8 layered, at places even thicker. The order and behaviour of the various layers during subsequent stages are as follows:

(i) Cells of the outer epidermis (*olii*) enlarge radially to form a palisade-like layer (*plii*). Except for some cells in the chalaza, the rest develop thickenings (Fig. 7 G, H). Cell enlargement as well as the thickenings start simultaneously from the micropylar and chalazal ends and progress towards the middle. Within the individual cells thickenings start from the outer side and extend towards the inside (Fig. 7 G). The contents of such cells are gradually lost.

(ii) The outer hypodermal layer (*ohii*) remains almost unchanged (Fig. 7 E–H).

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* The dye gets lost during processing and is not, therefore, observed in prepared slides.

FIG. 5 A–K. *Bixa orellana*, endosperm (*syn*—synergid; *z*—zygote). A, embryo sac showing primary endosperm nucleus and zygote. One of the synergids has degenerated while the other has enlarged. ×200. B–C, embryo sacs with 2 and 8 endosperm nuclei. Note the enlarged synergid. ×200. D, sixty-four-nucleate endosperm. ×102. E, non-synchronous divisions in the endosperm nuclei. The zygote has not yet divided. ×55·6. F, chalazal portion of an endosperm showing variation in the nuclear size. ×407. G, embryo sac showing initiation of cell formation in the endosperm at the micropylar end. Some of the endosperm cells have got detached from the tip of embryo sac. ×24·5. H, same, cell formation has extended up to the chalazal end. ×24·5. I, advanced stage of cellular endosperm, the central region has not yet been filled. ×17·9. J, enlarged view of peripheral portion of endosperm at the young cotyledonary embryo stage. ×407. K, portion of endosperm from a mature seed. The cells contain abundant starch grains. ×437.

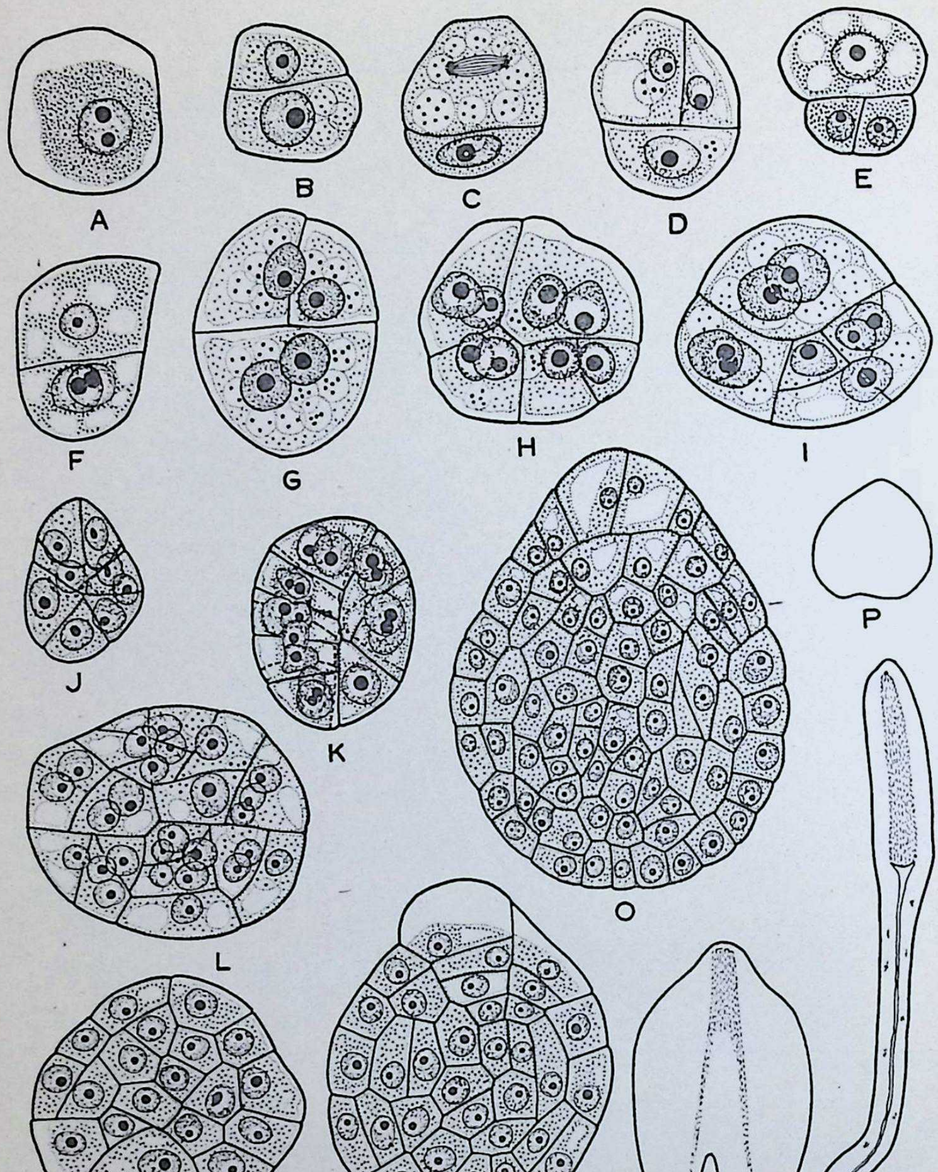

FIG. 6 A–R. *Bixa orellana*, embryo. A, zygote. ×868. B–C, two-celled proembryos; in C the nucleus in the basal cell is in division. ×868. D–G, three- and four-celled proembryos formed by a vertical division of the apical and basal cells of the two-celled proembryo. ×868. H–J, eight-celled proembryos. Note the difference in size and variation in the plane of divisions. ×865. K–O, young and old globular embryos. M and N show one enlarged cell each. K, M, ×868; the rest, ×704. P–R, diagrams of embryos (in l.s.) in later stages of development. P, Q, ×123; R, ×16.

(iii) The next 3–4 parenchymatous layers (*mlsii*) get greatly compressed, but towards the chalaza they cut off a number of derivatives (*dmlsii*; Fig. 7 D–H).

(iv) The cells of the inner hypodermal layer (*ihlii*; Fig. 7 G) develop thickenings and become hourglass-shaped (*hgc*; Fig. 8 H; *see also* Netolitzky 1926).

(v) Repeated divisions at the distal end of the inner epidermis (*ilii*) result in a massive tissue (*dilii*; Fig. 7 G, H).

Besides the above changes, various cell layers towards the micropylar end show radial elongation (Fig. 7 C) and there is a gradual deposition of tannin (*tfc*) in various parts of the seed (Fig. 7 A–H).

Pericarp

At the time ovular primordia are initiated, the ovary wall comprises 15–16 layers of homogeneous cells. During subsequent stages the number of layers increases and the cells enlarge, excepting those of the outer and inner epidermis. Dye cells are usually confined to a few layers below the outer epidermis. Mucilage cavities* develop inner to the vascular bundles and are initially arranged in more or less concentric rings. In the mature fruit tannin is deposited, especially in the cells surrounding the mucilage cavities and vascular bundles. Some of the inner layers of pericarp get detached and form a stiff membranous covering.

Ontogeny of mucilage cavities.—Certain cells in the pericarp enlarge and become densely cytoplasmic. They divide and redivide to form small groups of prominent cells. Later, degeneration starts in the central cells in each group and extends towards the periphery. The space thus formed enlarges and gets filled with mucilage.

Appendages on the fruit wall.—Two kinds of appendages occur on the fruit wall: (i) The vascularized outgrowths, which persist in the mature fruit as long stiff bristles and (ii) the non-vascularized appendages,† which occur on both sides of the pericarp and also on the vascularized appendages. The second type originates from the epidermal initials which enlarge and undergo longitudinal division. The resulting cells usually again divide longitudinally, at times transversely. Further divisions result in an expanded tip. Eventually, these appendages get detached from the ovary wall.

DISCUSSION

The various orders in which Bixaceae have been placed vary in their composition. Some families like Cistaceae and Cochlospermaceae are common to all, whereas others like Tamaricaceae, Flacourtiaceae and Frankeniaceae are present in most of them.

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* Mucilage cavities occur abundantly in almost all parts of the plant.

† These appendages also occur on other floral parts and on the inner side of cotyledons.

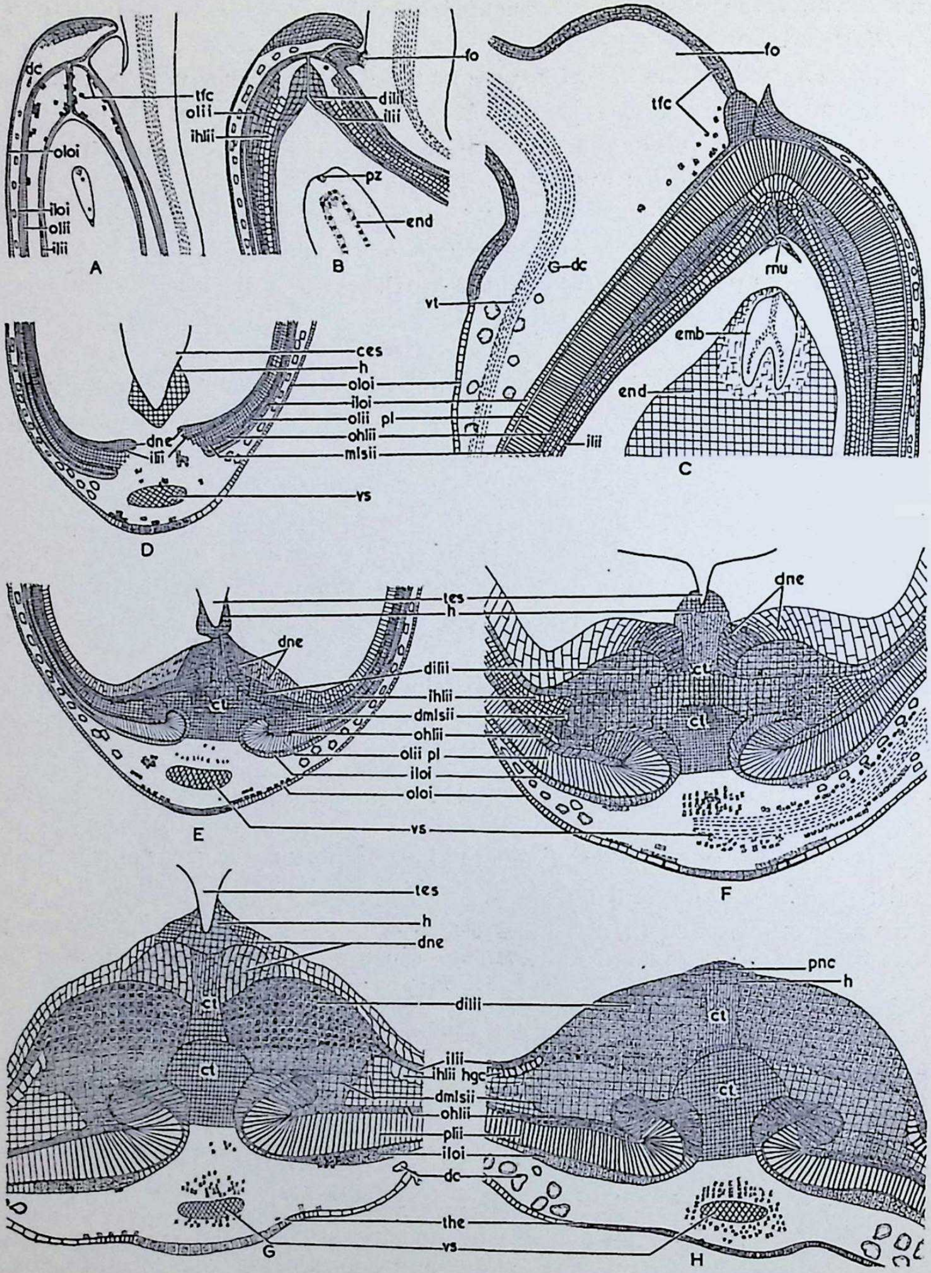
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Fig. 7 A–H. *Bixa orellana*, seed coat (*ct*—chalazal tissue; *dc*—dye cell(s); *dilii*—derivatives of inner layer of inner integument; *dmlsii*—derivatives of middle layers of inner integument; *dne*—derivatives of nucellar epidermis; *emb*—embryo; *end*—endosperm; *fo*—funicular outgrowth;

The Bixales of Hutchinson (1959) include seven families, but embryological data are available only on four of them. A comparison of their characters have been made in Table I.

The above data indicate affinities of Bixaceae with Cistaceae and Cochlospermaceae. Seed structure in these families resembles, especially in the formation of palisade-like layer from the inner integument and the characteristic curvature of the integuments in the chalazal region. Erdtman (1952) also stressed the affinities of Bixaceae with Cistaceae and Cochlospermaceae on the basis of his palynological observations.

Of the seven families included in the Bixales of Johansen (1950), four have been included in Table I, the 5th, Samydaceae, has not been worked out, and the 6th, Canellaceae, has recently been removed to the Ranales on the basis of anatomical and embryological features (*see* Parameswaran 1962; Wilson 1965). The 7th family, Frankeniaceae, differs from Bixaceae in having the following characters (*see* Walia and Kapil 1965):

(*a*) sepals 5, united, coriaceous, tubular
(*b*) petals 5, free, clawed
(*c*) anthers 5, free or connate at the base
(*d*) absence of parietal cells in the ovule
(*e*) formation of micropylar endosperm haustoria
(*f*) Solanad type of embryogeny.

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h—hypostase; *hgc*—hourglass-shaped cells; *ihlii*—inner hypodermal layer of inner integument; *ilii*—inner layer of inner integument; *iloi*—inner layer of outer integument; *mlsii*—middle layers of inner integument; *ohlii*—outer hypodermal layer of inner integument; *olii*—outer layer of inner integument; *oloi*—outer layer of outer integument; *plii*—palisade-like layer of inner integument; *pnc*—persisting nucellar cells; *rnu*—remnants of nucellus at the tip; *tes*—tip of embryo sac; *tfc*—tannin-filled cells; *thc*—thickened cells of chalaza; *vt*—vascular trace; *z*—zygote). A, diagram of upper portion of an ovule at mature embryo sac stage. ×86. B, diagram of the upper portion of a young seed showing cell enlargement in various layers. Note the initiation of outgrowth at the base of funiculus. ×71. C, diagram of upper portion of a seed at early cotyledonary stage of the embryo. The tannin-filled layers and the palisade layer have undergone further elongation. The funicular outgrowth has enlarged considerably. ×37·8. D, diagram of lower portion of young seed. ×71. E, same, at a later stage. The derivatives of nucellar epidermis have enlarged. In the inner integument, the innermost and the middle layers have proliferated; the outermost layer shows radial extension and incurving along with the innermost layer of the outer integument. ×46. F, same, at early globular embryo stage. The cells of the palisade layer have enlarged further and the curvature of this layer has become more pronounced. In the curved region the cells of the inner epidermis of the outer integument have also enlarged. ×46. G, same, at late globular stage of embryo. In the inner integument, the derivatives of the inner epidermis and middle layers have increased in bulk; cells of the palisade-like layer have become thick-walled. ×46. H, diagram of the lower portion of a mature seed. The hypostase is seen with a few persisting nucellar cells above it. Cells of the inner hypodermal layer of inner integument have developed into hourglass-shaped cells and the cells of the palisade-like layer have become thickened. The epidermal cells in the circular depression at the chalaza have become thick-walled. ×34·4. (The stippled areas in all the diagrams represent tannin-filled cells).

TABLE I

Character	Bixaceae (Mauritzon 1936; Venkatesh 1956; and present work)	Cistaceae (Chiarugi 1925; Souèges, 1937; Kapil and Maheshwari 1964)	Cochlospermaceae (Schnarf 1931)	Flacourtiaceae (Gopinath 1946; Narayanaswami and Sawhney 1959)
Anther	Horseshoe-shaped, basifixed, dehiscence by slits	Basifixed, dehiscence lengthwise	Dehiscence by apical pores	Dorsifixed
Archesporium	Single-celled	Multicelled	—	—
Tapetum	Secretory, 'Ubisch' granules present	Secretory, 'Ubisch' granules absent	—	Secretory, 'Ubisch' granules absent
Gynoecium	2-carpellary with 2 parietal placentae	3–5-carpellary, the placentae meeting in the centre to form axile placentation	3-carpellary, axile placentation	—
Ovule	Anatropous	Orthotropous	Anatropous, but after fertilization it curves and the raphe comes to lie on the inner side of the bend	Orthotropous, arillate, after fertilization the tip of the ovule bends like a hook
Archesporium	Single-celled	Multicelled	—	Single- or multicelled
Embryo sac	Polygonum type	Polygonum type	—	Polygonum type with variation in the functional behaviour of gaspores. The embryo sac becomes extra nucellar after 4-nucleate stage
Endosperm	Nuclear	Nuclear	Nuclear	Nuclear
Zygote	Does not elongate	Does not elongate	—	Elongates up to half the length of embryo sac

TABLE I—(*concld.*)

Character	Bixaceae (Mauritzon 1936; Venkatesh 1956; and present work)	Cistaceae (Chiarugi 1925; Souèges 1937; Kapil and Maheshwari 1964)	Cochlospermaceae (Schnarf 1931)	Flacourtiaceae (Gopinath 1946; Narayanaswami and Sawhney 1959)
Embryogeny	Irregular	Solanad	—	—
Seed	Albuminous, reserve material starch	Albuminous, reserve material starch	Albuminous, reserve material oil	—
Seed coat				
(a) Palisade layer	Present	Present	Present	—
(b) Hourglass-shaped cells	Present	Absent	Absent	Absent
(c) Dye cells	Present	Absent	Absent	Absent
(d) Any other behaviour	—	—	—	In the upper region the inner layer of inner integument becomes nutritive in function

TABLE II

Character	Bixaceae	Tamaricaceae (Battaglia 1941; Johri and Kak 1954)
Ovule	Anatropous, micropyle is formed by both the integuments	Anatropous, micropyle is formed by the inner integument only
Seed	Chalazal end highly modified	Hairy outgrowths at the chalazal end
Female gametophyte	Monosporic, Polygonum type	Tetrasporic, all types
Synergid polyembryony	Absent	Present in *Tamarix* and *Myricaria*
Embryogeny	Irregular	Solanad type
Suspensor	Absent	Forms a massive structure
Seed	Albuminous	Exalbuminous

Another family which is often placed close to Bixaceae is Tamaricaceae. The important embryological features of these families are presented in Table II.

The above data reveal that Tamaricaceae have no affinity with Bixaceae.

Metcalfe and Chalk (1950) suggested that anatomically Bixaceae show close relationship with Cochlospermaceae and Tiliaceae. Embryological features of Bixaceae and Tiliaceae are compared in Table III.

The above data, though insufficient, do not indicate any relationship of Tiliaceae with Bixaceae.

It thus appears, as Engler (1964) has also remarked, that differences in these families may prompt someone to raise them to an ordinal rank. As a matter of fact some of the orders, viz. Cistales, Tamaricales, etc., already exist in the literature. My views are in agreement with those of Engler who points out that such a classification would be of little use in the study of the existing links.

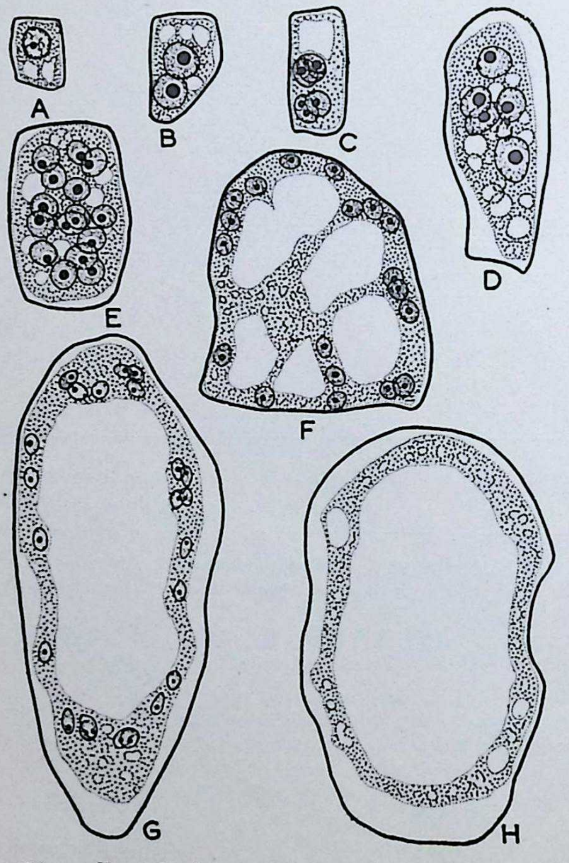

FIG. 8 A–H. *Bixa orellana*, development of dye cells. A–G, stages in the development of a dye cell. G, ×166; the rest, ×294. H, mature dye cell in which the nuclei have completely degenerated and disappeared. The dye has leached out during processing. ×166.

TABLE III

Character	Bixaceae	Tiliaceae (Banerji 1932; Venkata Rao and Sambasiva Rao 1952; Venkata Rao 1954; Bansal 1956)
Stamens	Numerous, all free	Numerous, members of the outer whorl absent or reduced to staminodes, monoadelphous or polyadelphous
Microsporangium	Single archesporial cell	Multicelled archesporium
Pollen grains	Round, 3-colporate, sexine thinner than nexine	Ellipsoidal, 3-colporate with sexine as thick as nexine
Megasporogenesis	Mostly single-celled archesporium	Multicelled archesporium, out of which only one functions
Obturator	Absent	Ovarian hairs constitute the obturator
Embryogeny	Irregular	Asterad type

As far as the grouping of families is concerned, the lack of sufficient embryological information prevents one from passing any conclusive remarks.

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